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(21) International Application Number: PCT/US96/09927 (22) International Filing Date: 7 June 1996 (07.06.96) (30) Priority Data: 08/480,662 7 June 1995 (07.06.95) US (60) Parent Application or Grant (63) Related by Continuation US 08/480,662 (CIP) Filed on 7 June 1995 (07.06.95) (71) Applicant (for all designated States except US): THE GOV- ERNMENT OF THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; Office of Technology Transfer, National Institutes of Health, Suite 325, 6011 Executive Boulevard, Rockville, MD 20852-3804 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): PASTAN, Ira [US/US]; 11710 Beall Mountain Road, Potomac, MD 20854 (US).		BRINKMANN, Ulrich [DE/US]; 4203 Brookfield Drive, Kensington, MD 20895 (US). (74) Agent: ALTMAN, Daniel, E.; Knobbe, Martens, Olson and Bear, 16th floor, 620 Newport Center Drive, Newport Beach, CA 92660 (US). (81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: CELLULAR APOPTOSIS SUSCEPTIBILITY PROTEIN (CSP) AND ANTISENSE CSP		
(57) Abstract The cDNA and amino acid sequences for a cellular apoptosis susceptibility (CAS) protein are used to detect expression and amplification of the CAS gene in normal and cancer cells. An antisense CAS gene sequence introduced into living cells inhibits CAS protein activity and thus prevents or inhibits apoptosis in the cells.		

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CELLULAR APOPTOSIS SUSCEPTIBILITY PROTEIN (CSP) AND ANTISENSE CSP**FIELD OF THE INVENTION**

The present invention relates to a novel gene sequence coding for a protein which is related to cell proliferation and programmed cellular death (apoptosis), and specifically relates to cDNA and amino acid sequences for this cellular
5 apoptosis susceptibility (CAS) gene and protein.

BACKGROUND OF THE INVENTION

Apoptosis or programmed cell death is a regulated network of biochemical events which lead to cell death. It is a physiological process involved in cell differentiation, organ development and maintenance of cellular populations in multicellular organisms (Cohen, J.J., *Immunol. Today* 14: 126-130, 1993). Furthermore, apoptosis is a reaction to various
10 external stimuli and cell damage (e.g. induced by drugs).

Apoptotic cells generally shrink and are phagocytosed by other cells. In contrast, necrotic cells are characterized by swelling, especially of the mitochondria which become dysfunctional, which usually results in cell lysis.

Molecular events characteristic of apoptotic cells include nuclear collapse with condensation of chromatin and loss of nucleoli. Later, the chromatin becomes fragmented into units of single or multiple nucleosomes which present
15 a "ladder" appearance when separated by size on a gel matrix (Compton, M.M., *Cancer Metast. Rev.* 11: 105-119, 1992). Activation of an endogenous endonuclease causes the chromatin fragmentation. Intracellular RNA, especially mRNA, is also degraded early during apoptosis.

Apoptosis can be triggered in various ways, including virus infection, growth factor withdrawal, DNA damage resulting from irradiation, exposure to glucocorticoids and certain chemotherapy drugs, or by signals such as TNF binding
20 to its receptor or crosslinking the Fas receptor with anti-Fas antibodies (Cohen, J.J., *Immunol. Today* 14:126-130, 1993; Williams, G.T., & Smith, C.A., *Cell* 74:777-779, 1993; Suda et al., *Cell* 75:1169-1178, 1993; Smith, et al., *Cell* 76:959-962, 1994; Lowe et al., *Nature* 362:847-849, 1993; Sentman et al., *Cell* 67:879-888, 1991). The mechanism of apoptosis is not well understood, but the observed molecular changes that occur in apoptotic cells suggest that endogenous genes are responsible for apoptosis. The proteins produced from these induced genes lead to destruction
25 of RNA and DNA ultimately leading to cell death.

Just as cell death by apoptosis is involved in normal regulation of cellular populations, cell proliferation is also required to maintain homeostasis of tissues and organs. However, in some cells, proliferation is aberrant leading to cancer. Cancer cells can be invasive, metastatic and highly anaplastic.

Although the mechanisms of tumor formation are still not completely and well defined, genetic elements,
30 including oncogenes, have been shown to increase cell proliferation and relieve cells of normal check-points in the cell division cycle. Genes that control cell cycle check points have been identified in multicellular organisms and in yeast where they play an essential role in regulating the cell cycle. It is even possible that cell cycle check points may serve as switch points for choosing between cell proliferation and apoptosis. Thus, when a gene that controls a checkpoint is deleted, mutated, amplified in the genome, or otherwise aberrantly expressed in the cell, it may divert the cell into
35 aberrant proliferation. Similarly, when a gene that normally controls a cell cycle switch point leading to apoptosis is aberrantly expressed, it may result in abnormal cell proliferation.

Some mammalian genes and proteins that have been simultaneously implicated in the regulation of cell proliferation and apoptosis including the genes for p53, BCL-2 or Myc. Although the pathways leading to apoptosis have not been fully elucidated, several genes that play a role in apoptosis have also been shown to play an important role in cancer. The p53 gene, coding for a tumor suppressor, is required for radiation-induced apoptosis (Lowe et al., *Nature* 5 362:847-849, 1993). BCL-2 inhibits apoptosis in many cells (Sentman et al., *Cell* 67:879-888, 1991; Vanhaesebroeck et al., *Oncogene* 8: 1075-1081, 1993) and furthermore, increased BCL-2 gene expression has been detected in primary breast cancer tissue without bcl-2 gene amplification (Nathan et al., *Ann. Oncol.* 5:409-414, 1994).

Apoptosis can also be induced by exposing cells to *Diphtheria* toxin (DT) or *Pseudomonas* toxin (PE) (Kochi, S.K., and Collier, R.J., *Exp. Cell Res.* 208: 296-302, 1993; Chang, M.P., et al., *J. Biol. Chem.* 264: 15261-15267, 1989; 10 Morimoto, H., and Bonavida, B., *J. Immunol.* 149: 2089-2094, 1992). Both of these bacterial toxins inhibit eukaryotic protein synthesis by inactivating elongation factor 2 by ADP-ribosylation (Carroll, S.F. and Collier, R.J., *J. Biol. Chem.* 262: 8707-8711, 1987). Although the toxin-specific mechanism by which these toxins induce apoptosis is unknown, it is not simply due to inhibition of protein synthesis because other protein synthesis inhibitors do not induce apoptosis (Chang, M.P., et al., *J. Biol. Chem.* 264: 15261-15267, 1989; Morimoto, H., and Bonavida, B., *J. Immunol.* 149: 2089- 15 2094, 1992).

SUMMARY OF THE INVENTION

According to one aspect of the invention, there is provided a purified and isolated cDNA coding for a human CAS protein having the sequence of SEQ ID NO:1.

According to another aspect of the invention, there is provided a purified and isolated cDNA sequence coding 20 for a portion of a human CAS protein consisting of nucleotides 2100 to 2536 of SEQ ID NO:1. In one embodiment, the cDNA sequence is an antisense CAS gene sequence corresponding to nucleotides 2100 to 2536 of SEQ ID NO:1

According to another aspect of the invention, there is provided a purified and isolated human CAS protein having of the amino acid sequence of SEQ ID NO:2.

According to another aspect of the invention, there is provided a purified and isolated human CAS protein 25 consisting of the amino acid sequence corresponding to amino acids at positions 700-845 of SEQ ID NO:2.

According to another aspect of the invention, there is provided a method of detecting cancerous cells, comprising measuring expression of a human CAS gene at a level higher than expression of a CAS gene in normal noncancerous human cells. In one embodiment of the method, the CAS gene expression is measured by detecting the level of CAS mRNA in cells. The CAS mRNA can be detected by hybridization with a complementary DNA or by a polymerase chain 30 reaction. In another embodiment, CAS gene expression is measured by detecting the level of CAS protein in cells. In a preferred embodiment, the CAS protein is detected by binding of antibody that specifically recognizes CAS protein.

According to another aspect of the invention, there is provided a method of detecting cancer cells, including measuring the number of copies of a CAS gene present in cells, wherein the number of copies of a CAS gene is higher in cancer cells than the number of copies of a CAS gene in normal noncancer cells. In one embodiment of the method, 35 the number of copies of a CAS gene is measured by detecting the amount of hybridization of a DNA complementary to SEQ ID NO: 1 or any portion of 25 or more nucleotides therein. In another embodiment, the method further includes

measuring the number of copies of a single-copy gene present in both cancerous and normal noncancerous cells and comparing the number of the single-copy gene to the number of copies of a CAS gene, wherein the number of copies of the CAS gene is higher than the number of copies of the single-copy gene. In one embodiment of the method, the number of copies of a CAS gene is determined by a polymerase chain reaction that amplifies either a 5' portion of the CAS gene, a 3' portion of the CAS gene, or both 5' and 3' portions of the CAS gene. In preferred embodiments of the method, the cancer cells are from breast, colon or lymph tissue. In one embodiment there is provided a kit for utilizing the method of this aspect of the invention, wherein the kit includes polynucleotides that serve as primers in a polymerase chain reaction for direct amplification of a nucleic acid coding for CAS protein and one or more reagents for performing a polymerase chain reaction or detecting a product of the polymerase chain reaction.

10 According to another aspect of the invention, there is provided a purified and isolated antibody that specifically recognizes CAS protein. One embodiment is a kit that utilizes the antibody for diagnosing cancer, including an antibody that specifically recognizes CAS protein and a means for indicating binding of the antibody to CAS protein.

According to another aspect of the invention, there is provided a method for preventing or reversing apoptosis in a mammalian cell, including the steps of providing the CAS cDNA sequence of SEQ ID NO:1 in a vector such that an antisense CAS transcript will be made in a cell under the regulation of DNA sequences contained within said vector, and administering an effective amount of the vector containing SEQ ID NO:1 to a mammalian cell thereby delivering the vector and CAS cDNA intracellularly.

According to another aspect of the invention, there is provided a method of treating cancer in a mammal in need of treatment, including administering to the mammal an agent that decreases CAS protein activity in cells, wherein the agent is an antibody that specifically recognizes CAS protein or an antisense CAS gene sequence that inhibits CAS protein activity by decreasing expression of a CAS gene. In a preferred embodiment, the agent is an antisense CAS gene sequence of the sequence of nucleotides 2100 to 2536 of SEQ ID NO:1 in a vector that includes genetic elements that regulate transcription of the antisense CAS gene sequence.

The foregoing general description and the following detailed description, with the accompanying drawings and examples, explain the principles of the invention and illustrate various embodiments of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a diagram of three plasmids showing the CAS cDNA clones in the "antisense" (FIG. 1A, pCDM/HE17) and "sense" (FIG. 1B, pCDM/HE17R) directions containing unrelated DNA (shadowed), and in the "antisense" direction (FIG. 1C, pCDM/HE17Δ) without the unrelated DNA.

30 FIG. 2 shows that CAS antisense reduces the sensitivity of MCF-7 cells to PE as determined by the MTS assay (FIG. 2A and 2B), even though protein synthesis is inhibited (FIG. 2C), and by time course experiments in which antisense transfectants undergo growth arrest upon toxin treatment but recover after removal of toxin (FIG. 2D - 2F).

FIG. 3 shows that the relative amount of CAS protein detected in MCF-7 cells containing the CAS antisense clone (MCF-7/17) was reduced compared to that detected in MCF-7 cells containing the CAS sense clone (MCF-7/R) and 35 control MCF-7 cells without a CAS clone (MCF-7/N).

FIG. 4 shows that CAS antisense renders MCF-7 cells less susceptible to apoptosis as determined by degradation of chromosomal DNA which was assayed by release of ³H-containing DNA.

FIG. 5 shows that CAS antisense reduces the sensitivity of MCF-7 cells to TNF as determined by: ³H-leucine incorporation into cells treated with TNF alpha (FIG. 5A and 5B) and TNF beta (FIG. 5C); by cell growth over 9 days after treatment with TNF alpha (FIG. 5D) and TNF beta (FIG. 5E); and by TNF alpha receptor binding (FIG. 5F).

FIG. 6 shows the DNA sequence of the CAS cDNA (FIG. 6A, SEQ ID NO:1) and the corresponding amino acid sequence (FIG. 6B; SEQ ID NO:2).

FIG. 7 shows the relative expression of CAS in human tissues and some cancer cell lines, as measured by Northern blots, from which the relative mRNA levels in tissues and tumor cell lines were determined.

FIG. 8A and 8B shows the copy number of the CAS gene detected in various cancer cell lines.

FIG. 9 shows the relative location of the CAS gene on 20q13.

FIG. 10 shows a hypothetical, schematic organization of typical eukaryotic gene to represent the CAS gene and depict PCR amplification of genomic CAS DNA using the combinations of primers represented by the numbers 51+52 and 31+32.

FIG. 11 shows the relative amount of CAS protein detected in growing and resting human WI-38 fibroblasts.

DETAILED DESCRIPTION OF THE INVENTION

Diphtheria toxin (DT) and *Pseudomonas* toxin (PE) are toxins that affect ADP-ribosylation that have been used to make recombinant immunotoxins that can specifically target and kill cancer cells *in vitro* and *in vivo* (Brinkmann, U., et al., *Proc. Natl. Acad. Sci. USA* 88: 8616-8620, 1991; Pastan I., et al., *Annu. Rev. Biochem.* 61: 331-354, 1992; Brinkmann U., and Pastan I., *Biochem. Biophys. Acta.* 1198: 27-45, 1994). However, it is known that for other anti-cancer agents, treated cells can become drug resistant causing the drugs to lose their efficacy (Schimke, R.T., *Cell* 37: 705-713, 1988; Gottesman, M.M., and Pastan, I., *Annu. Rev. Biochem.* 62: 385-427, 1993). Several known mechanisms by which cells can become resistant to ADP-ribosylating toxins, and thus possibly also to anti-cancer immunotoxins, include altered toxin binding, internalization, processing, and alterations in elongation factor 2 (EF2), the intracellular toxin target (Laurie and Robbins, 1991; Kido et al., 1991; Fendrick et al., 1992; Chaudhary et al., 1990).

We have used expression cloning to isolate cDNA-containing plasmids that cause breast cancer cells (e.g., cell line MCF-7) to become immunotoxin-resistant (Brinkmann, U., et al., *Molec. Med.* 1: 206-216, 1995). Using this approach, we isolated a gene that renders cells resistant to PE and DT. Because PE and DT induce apoptosis, the resistance of cells containing these clones suggested that the cloned cDNA was related to regulation of apoptosis. Hence we named the gene "CAS" for Cellular Apoptosis Susceptibility, in reference to the function of the protein product of the gene. Plasmids containing even a portion of the CAS gene rendered the MCF-7 cells resistance to native PE and DT, indicating that the resistance was due to interference with the action of the toxin moiety rather than another portion of the immunotoxins.

In cells transfected with the CAS clones, the cells did not die upon exposure to PE or DT, even though the toxins caused modification of EF2 and inhibition of protein synthesis comparable to toxin-sensitive control cells. Thus, these clones represent a class of genes that determine the sensitivity of cells to toxin after inhibition of protein synthesis,

the primary action of the toxins, has occurred. The isolated and purified cDNA coding for the CAS protein is the subject of this invention.

The clone that mediated PE- and DT-resistance of transfected MCF-7 cells, without interfering with ADP-ribosylation of EF2 and subsequent inhibition of protein synthesis, contains an antisense CAS cDNA fragment. MCF-7 cells containing the antisense CAS cDNA clone were less susceptible to toxin induced apoptosis and apoptosis induced by TNF-alpha and TNF-beta. These results show that the gene has a function in apoptosis. Independent from that function in apoptosis, we found that expression of the human CAS gene was elevated in tissues containing many dividing cells and in tumor cell lines, indicating that CAS also has a role in cell proliferation. The CAS gene sequence is partially homologous with that of the yeast CSE1 gene, which plays a role in yeast cell division (Xiao, Z., et al., *Mol. Cell. Biol.* 13: 4691-4702, 1993).

The present invention includes a complete cDNA sequence coding for CAS protein and the corresponding complete amino acid sequence of CAS protein. The sequences are useful for making purified CAS protein which can be used to develop protein-based kits for detection of CAS protein, a potential marker for cancer, in human cells and tissue. The DNA sequence, in whole or part, can be used as a probe to detect gene expression and genetic rearrangements of the CAS gene and the surrounding chromosomal region. This is useful for diagnosing cancer and determining its degree of malignancy based on CAS gene expression or genetic rearrangement. This aspect is especially important in diagnosing and treating cancer by indicating the physiological condition of cancerous cells thus allowing a clinician to administer an appropriate treatment for the detected cancer. The sequence may be useful as an antisense therapeutic composition for decreasing functional CAS expression intracellularly and thus controlling growth of malignant cells or tissue. The sequence may also allow one to induce abnormal physiological conditions in a mammal by overexpressing the CAS gene to create animal models for human pathological conditions (e.g., breast and colon cancer) which are useful for discovery of new therapeutics to improve the current treatments of these conditions.

A clone containing a CAS cDNA was isolated from a cDNA library utilizing a selection for immunotoxin resistance in cells transfected with the CAS cDNA clone. That is, an immunotoxin in which a truncated form of the toxin PE is joined to the variable region of an antibody that recognizes cellular marker molecule is used to select stably transfected cells that survive incubation with the immunotoxin. The immunotoxin kills cells by delivering a toxin that causes cell death by inactivating EF2 by ADP-ribosylation of the elongation factor, thereby arresting protein synthesis in the cells. Clones from a library of human cDNA molecules that are expressed from a promoter and other translational signals were stably transfected into immunotoxin-sensitive recipient cells using any of a variety of transfection methods including electroporation. Cotransfection of additional plasmids to provide other selectable drug resistance markers or expression signals for transcription and/or translation of the transfected human cDNA may also be used. The recombinant cell lines were then selected for their immunotoxin resistance, either as stable clones or as transiently expressing clones.

Using this approach, a transfected cell line containing a clone that made the transfected cells about ten-fold less sensitive to the PE-derived immunotoxin was isolated. The cells were also demonstrated to be resistant to native PE and DT, but were not resistant to ricin or cycloheximide, drugs that inhibit protein synthesis by other mechanisms.

The clone that conferred this PE and DT resistance contained a cDNA that was transcribed to produce an antisense RNA molecule. The sequence of the cDNA was determined using standard DNA sequencing procedures and revealed a cDNA fragment of a previously unidentified human homolog of the yeast gene CSE1, a chromosome segregation gene. The human gene was designated CAS for "cellular apoptosis susceptibility" because subsequent experiments showed that the gene was involved in the process of apoptosis. Unrelated sequence contained in the cDNA clone was also revealed by DNA sequencing but was a cloning artifact because the unrelated sequence was not found associated with a full length CAS clone subsequently isolated and did not contain an open reading frame nor was it expressed in the RNA of any human tissue tested by Northern blotting.

The antisense CAS cDNA was responsible for conferring toxin resistance to the transfected cells because a subclone containing the CAS cDNA in an orientation that produces sense RNA did not render the cells toxin resistant. The PE-resistance of cells containing CAS cDNA in an orientation that produces antisense CAS RNA was confirmed in assays measuring cell proliferation over multiple days. In those assays, the cells containing antisense CAS constructs demonstrated growth arrest upon toxin treatment but maintained cell viability and recovered showing cell proliferation when the toxin was removed, reaching about the same cell number as the control cells that were not PE treated after a few days of resumed growth. In contrast, control cells treated with PE died as a consequence of toxin treatment. The viability of cells containing antisense CAS was confirmed by morphological observations in which the CAS antisense containing cells maintained a viable appearance (flattened and attached to the substrate) during PE treatment whereas control cells detached from the substrate and began to disintegrate.

Cells containing antisense CAS cDNA constructs, like control cells, have protein synthesis inhibited by inactivation of EF2 as demonstrated by an assay that measures incorporation of radioactivity into protein. The PE-mediated alteration of EF2 was directly confirmed in an ADP-ribosylation assay. Thus, the CAS antisense did not make cellular EF2 resistant to PE but mediated cellular resistance to the toxin by another mechanism. This is related to CAS protein production in the transfected cells because the antisense CAS constructs reduced the level of CAS protein about 65% compared to controls, as detected by immunoblotting assays in which anti-CAS polyclonal antibodies specifically detected CAS protein of about 100 kDa.

Nuclear DNA degradation associated with toxin-induced apoptosis was decreased in cells containing antisense CAS constructs. The nuclear DNA degradation resulting from PE treatment of control cells that did not have antisense CAS released DNA fragments into the culture medium and produced DNA with a characteristic "ladder" appearance (resulting from internucleosomal DNA degradation) when visualized on a gel. In contrast, antisense CAS containing cells treated with PE showed considerably less soluble DNA in the medium and showed only a very faint "ladder" pattern with most of the DNA remaining in high molecular weight form of greater than 1500 bp. These results suggest that CAS antisense produces toxin resistance by interfering with the apoptosis pathway. Hence, the presence of CAS protein makes cells susceptible to apoptosis.

Because apoptosis is known to be induced in cells exposed to tumor necrosis factors (TNF) α and β , the effect of CAS antisense was determined in transfected cells containing CAS antisense constructs. All of the cells tested were shown to have TNF receptors using binding assays to detect binding of radiolabeled TNF α . Control cells were about

5-fold to 10-fold more sensitive to both $TNF\alpha$ and $TNF\beta$ than cells containing antisense CAS as determined by an assay to measure radioactivity incorporation into proteins. This CAS antisense mediated resistance to $TNF\alpha$ and $TNF\beta$ was confirmed by morphological observation of the treated cells, which remained normal compared to the killed control cells. Cell proliferation assays also confirmed that CAS antisense transfected cells were growth inhibited by TNF treatment but the inhibition was reversed when TNF was removed.

Using the cDNA clone containing 436 bp of CAS cDNA as a probe, longer clones containing up to 2.4 kb of CAS cDNA were isolated from a human cDNA library; these clones all contained 3' end CAS cDNA. A full length CAS cDNA clone was expected to be about 3.2 kb long based on the size of CAS mRNA detected in Northern blots of RNA obtained from human cell lines. To isolate a full length CAS cDNA clone, polymerase chain reaction amplification of the 5' portion of the CAS cDNA (a 1160 bp fragment) from placenta cDNA was used to produce a 5' CAS cDNA fragment that overlapped the 3' CAS cDNA clone. From these clones, the complete CAS cDNA sequence was determined (SEQ ID NO:2) and the amino acid sequence of the CAS protein was deduced (SEQ ID NO:2). When the coding regions of the human CAS gene and the yeast CSE1 gene were compared, their sequences were about 59% homologous overall and a search of DNA and protein databases revealed no other defined protein with significant homology to CAS (Brinkmann, U. et al., *Proc. Natl. Acad. Sci. USA* 92:10427-10431, 1995).

CAS gene expression in a variety of normal human tissues and human cancer cell lines was demonstrated using Northern blotting. The CAS mRNA (about 3 kb in length), was found in RNA from pancreas, adrenal medulla, thyroid, adrenal cortex, testis, thymus, small intestine, stomach, spleen, lymph node, thymus, appendix, peripheral blood, bone marrow, fetal liver, spleen, prostate, ovary, colon, heart, brain, placenta, lung, liver, skeletal muscle and kidney tissues, as well as tumor cell line RNA from a variety of cell lines. Quantitation of CAS expression, normalized to the expression of actin or GAPDH, showed that relative expression of CAS was high in tissue containing many actively proliferating cells (e.g., testis and fetal liver), and was elevated above basal level in tissues that contain some proliferating cells (e.g., lymphoid tissues). Thus, in addition to its role in apoptosis, CAS expression is associated with cell proliferation.

Anti-CAS polyclonal antibodies were produced in animals immunized with purified recombinant CAS proteins containing amino acids 1-284 and 327-669 of the complete CAS amino acid sequence. The anti-CAS specific antibodies were purified using standard techniques and used to detect CAS protein on immunoblots of gel separated cellular proteins, detecting a single band of about 100 kDa. The anti-CAS antibodies have been used to determine the relative expression of CAS protein in resting (normal) cells compared to proliferating and tumor cells, and to determine the cellular location of CAS protein.

Because CAS mRNA is highly expressed in proliferating cells and tumor cell line, proteins in cell extracts made from growth arrested and actively growing human cells were analyzed for production of CAS protein. Growth arrested cells that were serum starved produced considerably less CAS protein than actively growing cells indicating that elevated CAS protein is a marker for cell proliferation. When proteins extracted from breast cancer cells were compared to normal (fibroblast) cell proteins, elevated levels of CAS protein were detected by immunoblotting showing that CAS is expressed in significantly higher levels in cancer cells, thus indicating that anti-CAS antibodies are useful for diagnosing cancer by detecting elevated CAS levels in cancer cells. Such an anti-CAS antibody based diagnostic kit may utilize any of a

variety of known immunoassays including but not limited to fluorescently-labeled antibody detection, radioimmunoassay (RIA), colorimetric antibody detection such as using antibody linked to latex beads or an enzyme linked immunosorbent assay (ELISA), and a sandwich immunoassay involving multiple reagents.

The cellular location of CAS protein was also determined using anti-CAS antibodies in immunoblotting analyses.

- 5 The CAS protein is a relatively abundant protein (about 600,000 molecules per cell) found in the cytosol. Using immunofluorescent labeling of cellular substructure, the CAS protein was associated with microtubules, similar to the location of the protein tubulin, and in mitotic cells CAS is associated with the mitotic spindle (Scherf, U. et al. *Proc. Natl. Acad. Sci. USA* 93:2670-2674, 1996). These immunofluorescent labeling results confirmed that CAS is present in elevated amounts in proliferating cells including testicular spermatogonia, basal cells of the colon, and in the respiratory
- 10 epithelium of the trachea and in axons and Purkinje cells of the cerebellum. Using anti-CAS antibodies in immunostaining analysis of formalin-fixed tissues, these results were extended to lymphoid tissue found in normal tonsil tissue and lymphomas. CAS protein was detected in tonsil tissue, primarily in cytoplasm of large cells of the dark zone of the follicle but not in mantle zone cells or interfollicular cells. In many different types of lymphomas, CAS protein was also found in many cells, generally correlating with the distribution of another cellular proliferation protein marker (Ki-67), and
- 15 found predominantly in the larger malignant cells of the tumors, thus showing that CAS is useful as a marker for cancer cells.

- Utilizing Southern blotting techniques with a CAS genetic probe and and fluorescent *in situ* hybridization with a CAS-containing P1 clone, the CAS gene was localized to human chromosome 20 in the 20q13 region, a region that is known to be amplified in some breast cancers, colon and bladder cancer cell lines (Brinkmann, U. et al., *Genome Res.*
- 20 6:187-194, 1996). Quantitative Southern blotting was used to determine that the CAS gene was amplified (two- to eight-fold) compared to a single-copy gene (e.g., actin) in a number of cancer cell lines, particularly in breast cancer, colon cancer and leukemia lines. More detailed information on the genetic amplification of the CAS gene was obtained using Fluorescent In Situ Hybridization (FISH), comparing CAS in cancer cell lines to the CAS gene detected in normal human peripheral blood lymphocytes (PBL) which have two copies of the CAS gene and 46 chromosomes per cell. Many of the
- 25 tested breast and colon cancer cell lines and one leukemia showed amplification of the CAS gene associated with some chromosomal abnormalities, such as an extra copy of chromosome 20, an aberrant chromosome 20 such as no normal chromosome 20 but detection of 20q marker chromosomes, an amplification of the 20q region, or a CAS-specific amplification and translocation to another chromosomal region than 20q. Other chromosomal abnormalities (e.g., polyploidy) were also detected in the cancer cell lines. Thus, the CAS gene serves as a useful marker of chromosomal
- 30 abnormalities in chromosome 20, particularly in the 20q region, associated with breast and colon cancers and leukemia.

- A complete genomic clone of the CAS gene was isolated from a human genomic library and the presence of the 5' and 3' ends of the CAS gene on the clone were confirmed using PCR amplification of the ends of the CAS gene, using primers based on the sequence of the CAS cDNA at the 5' and 3' ends. A similar PCR-based assay using primers specific for the CAS gene sequence is useful for detecting amplification of CAS gene sequences in DNA isolated from
- 35 human tissue as the template. In this PCR-based assay, genomic DNA is isolated from tissue to be tested (e.g., a biopsy of breast tissue) and the DNA is used as a template for a PCR amplification of either a 5' end, a 3' end or both 5' and

3' ends of the CAS gene using appropriate combinations of primers specific for amplifying the respective end(s) of the CAS gene. The PCR amplification of the CAS gene is compared to a PCR amplification of a known single-copy gene in human DNA (e.g., actin). Using appropriate calculations to normalize the amount of amplification of the portion of single-copy gene compared to the amplified portion(s) of the CAS gene (i.e., based on the relative sizes of the amplified gene portions), the number of copies of the CAS gene in the tissue is approximated to serve as a diagnostic for cancer tissue and as a prognosticator of the aggressiveness of the cancer if cancer is detected. For example, about a two-fold amplification of the CAS gene relative to the control single-copy gene in the tissue DNA indicates that there is probably a 20q chromosomal abnormality in the tissue DNA, indicative of cancer. Additional amplification of the CAS gene detected in the tissue DNA (e.g., greater than two-fold) is an indicator of an aggressive cancer which is used by the treating physician in making a prognosis and deciding on appropriate therapy.

The general principles of the present invention may be more fully appreciated by reference to the following examples.

EXAMPLE 1: ISOLATION OF A cDNA CLONE THAT RENDERS A BREAST CANCER CELL LINE RESISTANT TO CELL DEATH INDUCED BY TOXINS

Expression cloning and immunotoxin selection of cDNA plasmids. A cDNA library in plasmid pCDM8 that contains HeLa cDNA expressed from a CMV promoter and followed by a SV40 polyA sequence was obtained from Clontech and transfected into MCF-7 breast carcinoma cells. Clones of human cDNAs that confer resistance to the immunotoxin B3(Fv)-PE38KDEL were isolated by expression cloning and selection (Brinkmann, U., et al., *Molec. Med.* 1: 206-216, 1995) with the immunotoxin B3(Fv)-PE38KDEL, a fusion protein composed of a truncated form of PE and the Fv region of monoclonal antibody (MAb) B3 that binds to a carbohydrate present on many carcinomas and cancer cell lines, including MCF-7 cells, and kills them (Brinkmann, U., et al., *Proc. Natl. Acad. Sci. USA* 88: 8616-8620, 1991).

Briefly, MCF-7 cells expressing the SV40 T antigen, which allows episomal replication in the cells, (MCF-7/T; Brinkmann, U., et al., *Molec. Med.* 1: 206-216, 1995) were transfected with a HeLa cDNA expression library and treated two days later with high doses of B3(Fv)-PE38KDEL (Brinkmann, U., et al., *Proc. Natl. Acad. Sci. USA* 88: 8616-8620, 1991). Dead cells were removed by washing with phosphate buffered saline (PBS), the remaining cells harvested, and plasmids were recovered (Hirt, B., *J. Mol. Biol.* 26: 365-369, 1967). The recovered plasmids were propagated and amplified in *E. coli* (strain MC1061/P3) using 35-50 μ g/ml ampicillin and 12.5-15 μ g/ml tetracycline. Plasmid DNA for transfections was purified by standard techniques well known in the art based on elution from DEAE-Sephacel (using a Qiagen™ "Mega" plasmid DNA preparation kit) and retransfected into MCF-7/T for two additional rounds of immunotoxin-selection and plasmid reisolation as described above. Using this procedure, plasmids that caused cells to survive after immunotoxin exposure were selectively enriched resulting in siblings of the same or overlapping cDNAs in the final plasmid pool analyzed. Such plasmids were identified by DNA hybridization analysis and several plasmids were found that were present more than once in a pool of 96 individual plasmid clones randomly isolated after three rounds of immunotoxin selection.

These toxin-resistance clone candidates were stably transfected into MCF-7 cells and analyzed individually for their effects on toxin sensitivity (as described in Brinkmann, U., et al., *Molec. Med.* 1: 206-216, 1995). Cell line MCF-7/17 that contains a plasmid, pCDM/HE17, (called "p17" in Brinkmann, U., et al., *Molec. Med.* 1: 206-216, 1995) was obtained by cotransfection with another plasmid, pMC1neo/polyA, that confers resistance to the drug G418 which was used to select transformants using standard tissue culture methods.

Molecular manipulations of clones. pCDM/HE17 was isolated from that library by immunotoxin selection and contains a 700 bp insert composed of 436 bp of antisense cDNA called CAS and 264 bp of unrelated sequence (FIG. 1). The CAS gene was identified as an open reading frame with homology to a yeast gene, CSE1 (Xiao, Z., et al., *Mol. Cell. Biol.* 13: 4691-4702, 1993). Standard cloning techniques (Sambrook, J., et al., *Molecular cloning. A Laboratory Manual*, 2nd ed., 1989) were used to make pCDM8/HE17R, a plasmid containing the 700 bp cDNA in an inverse orientation, and pCDM8/HE17Δ, a plasmid in which a 260 bp Asp700-HindIII fragment that is not homologous to a CSE1 sequence has been deleted.

The relevant portions of these three plasmids are diagrammed in FIG. 1. Plasmid pCDM/HE17 contains a 700 bp insert composed of 436 bp antisense cDNA (open box), which is homologous to the yeast CSE1 gene, and 264 bp unrelated sequence (shadowed box) in an antisense direction behind the CMV promoter followed by the SV40 poly-A signal. X, A and H indicate XbaI, Asp700 and HindIII sites. pCDM/HE17R contains the cDNA (XbaI-fragment) of pCDM/HE17 in reverse orientation and pCDM8/HE17Δ has the 260 bp Asp700-HindIII non-CSE1 fragment deleted. As negative controls, four plasmids containing unknown inserts (pCDM8/C2-pCDM8/C5) were randomly chosen from the same library.

Recombinant MCF-7 cell lines. Cell lines were produced by electroporation of 5×10^6 MCF-7 cells with 3 μg pMC1neo/polyA and 15 μg of expression plasmid, using a Biorad™ gene pulser at 400 V, 960 FD, in 0.4 cm cuvettes (as described in Brinkmann, U., et al., *Molec. Med.* 1: 206-216, 1995). MCF-7/T cells are a pool of MCF-7 cells that express SV40 large T antigen, thus allowing episomal replication of plasmids with an SV40 origin (e.g., pCDM8). MCF-7/T cells were made by cotransfection of pCMV-Tag (Ogryzko, V.V., et al., *J. Virol.* 68: 3724-3732, 1994) and pMC1neo/polyA and selection of a transfected cell pool with 0.8 mg/ml G418 (FIG. 1A). Similarly, MCF-7/17, MCF-7/17R and MCF-7/17Δ cell pools were produced by cotransfection of pMC1neo/polyA and 15 μg of pCDM/HE17, pCDM/HE17R or pCDM/HE17Δ, respectively. MCF-7/C and MCF-7/C2-C5 cells were produced by cotransfection of pMC1neo/polyA with pCDM8 (vector alone, to produce MCF-7/C cells) or pCDM/C2-C5 (to produce MCF-7/C2-C5 cells), control plasmids randomly chosen from the library without any selection. MCF-7/N cells contained only pMC1neo/polyA. All these cell lines were selected and propagated with media containing 0.8 mg/ml G418 and were not exposed to toxins or TNF until their response to these agents was tested.

One clone that conveyed immunotoxin resistance on MCF-7 breast cancer cells is pCDM/HE17, a plasmid containing a 700 bp HeLa cDNA insert (FIG. 1). This clone rendered MCF-7 cells about 10-fold less sensitive to a PE-derived immunotoxin as well as to native PE and DT (Brinkmann, U., et al., *Molec. Med.* 1: 206-216, 1995). Both toxins usually cause cell death by inactivating EF2 (by ADP-ribosylation of the protein), thereby arresting protein synthesis. Although cells containing clone pCDM/HE17 were resistant to PE and DT (about 10-fold compared to control

MCF-7/N cells, they were not resistant to ricin or cycloheximide (having a LC_{50} of about 250 ng/ml), which inhibit protein synthesis by other mechanisms (Brinkmann, U., et al., *Molec. Med.* 1: 206-216, 1995), nor are they protected against cell death induced by various chemotherapeutic agents, e.g., VP 16 (having a LC_{50} of 30 μ g/ml) and staurosporine (having a LC_{50} of 1-2 ng/ml).

5 The cDNA insert in plasmid pCDM/HE17 was determined using fluorescently labeled dideoxynucleotides essentially according to the method of Sanger et al. (*Proc. Natl. Acad. Sci. USA* 74: 5463, 1977) using an automated sequencer (ABI™ model 373A and the ABI™ Dye-deoxy Terminator kit).

The DNA sequence of pCDM/HE17 cDNA revealed a 436 bp cDNA fragment that is part of a previously unidentified human homolog of the yeast CSE1 chromosome segregation gene (Xiao, Z., et al., *Mol. Cell. Biol.* 13: 4691-10 4702, 1993), which we call CAS. The human CAS cDNA clone has 45% protein identity and 66% similarity of amino acids when compared to the yeast CSE1 sequences (Xiao, Z., et al., *Mol. Cell. Biol.* 13: 4691-4702, 1993) in this portion of the DNA (discussed in detail in Example 9). Plasmid pCDM8/HE17 also contains 264 bp of an unrelated sequence fused to CAS sequences. This is library-ligation artifact because this sequence is not related to the sequence of the full length CAS isolated from human placenta cDNA (discussed in Example 7) and also not linked to the gene in HeLa cells, 15 as shown by hybridization of Southern blots of HeLa and placenta DNA cleaved with various enzymes. This unrelated sequence does not have any open reading frame and therefore is noncoding, and its expression was not detected by Northern blot analysis of RNA from any human tissue. The CAS clone is present in an "inverse" orientation so that transcription from the CMV promoter of pCDM/HE17 generates an antisense RNA. Therefore, the resistance mediated by pCDM/HE17 is likely due to antisense RNA produced from this partial CAS gene sequence which interferes with 20 expression of the human CAS gene.

EXAMPLE 2: CELLULAR RESISTANCE TO TOXINS IS MEDIATED BY CAS ANTISENSE RNA

To confirm that toxin resistance mediated by clone pCDM/HE17 is caused by antisense RNA production of CAS RNA, a plasmid (called pCDM/HE17R; see FIG. 1B) was constructed in which the insert of pCDM/HE17 was inverted so that sense RNA fragment would be made from the plasmid. This plasmid did not render cells resistant. To rule out a 25 possible effect of the additional non-CAS sequence, the 264 bp sequence that showed no homology to the yeast CSE1 gene was also deleted, to produce plasmid pCDM/HE17 Δ (FIG. 1). These plasmids were individually cotransfected with plasmid pMC1neo/polyA into MCF-7 cells and stably transfected cells containing pMC1neo/polyA and each of these plasmids were isolated. Additional controls were MCF-7 cells cotransfected with pMC1neo/polyA and the library vector pCDM8 without insert (called MCF-7/C), MCF-7 cells with randomly chosen plasmids from the unselected pCDM8/HeLa 30 library (called MCF-7/C2-C5), and MCF-7 cells with pMC1neo/polyA alone (called MCF-7/N). Cells were propagated using G418-resistant drug selection. Cells were not exposed to toxin until their response to toxins was analyzed. All transfectants (including the parent clone, MCF-7/17) were pooled transfectants (greater than 10 colonies) rather than isolated single clones to compensate for possible clonal variability in the cells containing the transfected plasmids (Brinkmann, U., et al., *Molec. Med.* 1: 206-216, 1995).

35 Figure 2 shows experiments in which the sensitivity to PE of MCF-7 cells and CAS antisense transfected MCF-7 cells was analyzed using the MTS cell proliferation assay. PE was chosen to represent ADP-ribosylating toxins (which

also include immunotoxins and DT) in this study because the relative sensitivities or resistance of the cell lines to PE, DT and immunotoxins were comparable, i.e., cells resistant to PE also were resistant to immunotoxin as well as to DT (Brinkmann, U., et al., *Molec. Med.* 1: 206-216, 1995).

Sensitivity to PE was assayed by MTS assays (Cory, A.H., et al. *Cancer Commun.* 3: 207-212, 1991) which
5 detect dehydrogenases present in living cells (using absorbance at 490 nm which is proportional to number of live cells), or by assaying incorporation of ³H-leucine into cellular proteins (protein synthesis inhibition assay; Brinkmann, U., et al., *Proc. Natl. Acad. Sci. USA* 88: 8616-8620, 1991). Usually 3×10^3 cells/well in 200 μ l medium were plated and grown overnight, incubated with toxin for 20 hrs (or as indicated), and then assayed. Referring to FIG. 2, the symbols are the same for all experiments. MCF-7/17 cells (○) and MCF-7/17 Δ cells (Δ) contained CAS antisense clones. Controls were
10 MCF-7/N cells (●) which contained only the pMC1neo/polyA plasmid and MCF-7/C cells (■) which contained pMC1neo/polyA and the pCDM8 library vector without insert. The MCF-7/17R cells (\blacktriangle) contained CAS cDNA in the sense direction (see FIG. 1 for diagrams of the plasmids with CAS inserts).

FIG. 2A shows an experiment in which cells were exposed to various concentrations of PE for 3 days and the number of cells present on day 3 was measured by the MTS assay. It is evident that MCF-7/17 cells containing CAS
15 antisense were about 10-fold more resistant to PE than the control cell lines (MCF-7/N) and MCF-7/C, with an IC₅₀ of about 15 ng/ml compared to 1-2 ng/ml in the control. To eliminate the possibility that the additional non-CAS sequence influences toxin sensitivity, MCF-7/17 Δ cells containing CAS antisense without the additional sequence were also tested.

Figure 2B shows that these cells were also more resistant to PE, although the resistance was not as
20 pronounced as with MCF-7/17. These experiments show that the presence of CAS antisense reduces the sensitivity of MCF-7 cells towards PE.

FIG. 2C shows inhibition of protein synthesis, assayed by measuring the incorporation of ³H-leucine 20 hrs after toxin addition. All cells tested showed the same amount of inhibition of protein synthesis indicating that the PE entered the cells and inactivated EF2, its normal cellular target.

25 FIG. 2D-F show time course experiments in which transfectants containing antisense CAS undergo growth arrest upon toxin treatment but recover after removal of toxin. Cell viability was determined by MTS assays in 96-well plates where A490 is proportional to the number of cells in each sample. MCF-7/17 cells, producing CAS antisense, showed growth about equal to that of the control cells, but a longer lag-phase before resuming growth after trypsinization. In FIG. 2E and 2F, a typical growth curve of cells without toxin (representing the mean of three experiments) is indicated
30 by a broken line and the duration of toxin exposure is indicated by a bar on the X-axis.

EXAMPLE 3: CELLS MADE RESISTANT TO TOXIN-INDUCED APOPTOSIS BY ANTISENSE CAS EXPRESSION STILL HAVE TOXIN-ALTERED ELONGATION FACTOR-2 (EF2)

The results shown in FIG. 2C indicated that PE altered EF2 in the treated cells whether or not the CAS gene was present or transcribed in the sense or antisense orientation. This was directly confirmed using an ADP-ribosylation
35 assay.

Extracts for assaying ADP-ribosylation of EF2 by PE were prepared by suspending PBS-washed cell pellets in lysis buffer (10 mM Tris, 10 mM KCl, 1.5 mM Mg-acetate, 6 mM mercaptoethanol, pH 7.5) for 30 min at 20°C followed by homogenization. Then 0.2 vol 1.25 M sucrose was added, the suspension was centrifuged (30 min, 4°C, 100,000 x g) and the supernatant was dialyzed against lysis buffer containing 0.25 M sucrose. Equal amounts of protein in 5 extracts were incubated in 250 μ l (final vol, adjusted with 50 mM Tris, 1 mM EDTA) assay buffer containing 40 mM DTT, with 1.5 μ l 14 C-NAD (Amersham, 287 mCi/ μ Mole, 260 μ Ci/ml) and 10 ng/ml PE (final conc.) for 30 min at 37°C. Protein-associated radioactivity was precipitated with 12% TCA, and then washed with 6% TCA. The pellet was solubilized with 0.1 M NaOH, neutralized with HCl and radioactivity was determined by counting disintegrations per min in scintillation liquid.

10 As shown in FIG. 2C, there was no difference in the incorporation of 3 H-leucine between MCF-7 cells containing CAS antisense and the control cell lines, even though the MTS assay showed that the toxin sensitivity was very different between these cell lines. In these experiments, an additional control cell line which contained CAS expressed in the sense direction (called MCF-7/R) was included; it showed identical results as the experimental cells for 3 H-leucine incorporation after PE treatment.

15 The direct measurement of the ability of PE to ADP-ribosylate EF2 in extracts of CAS antisense MCF-7/17 cells and in control cells shows that the sensitivity of EF2 in the toxin resistant transfectant was indistinguishable from controls (see Table 1). Thus, CAS antisense does not make cellular EF2 resistant to PE. The EF2 becomes modified as effectively in cells containing CAS antisense as in control cells.

Table 1: The immediate actions of PE and TNF are not affected in CAS antisense containing cells (PE)

20			MCF-7, MCF-7/N (control)	MCF-7/17 (CAS antisense)
	PE	ADP-ribosylation activity (cpm)	1328 ± 250	1329 ± 200
		protein synthesis inhibition (IC ₅₀) (ng/ml)	0.5-1	0.5-1
		cell death (LC ₅₀) (ng/ml)	1-2	15-20
25	TNF	receptors/cell	17000 ± 4000	17000 ± 5000
		affinity (pM)	102 ± 8	104 ± 17
		cell death (LC ₅₀) TNF α (ng/ml)	0.15	1-3
		cell death (LC ₅₀) TNF β (ng/ml)	1-3	20-30

30 ADP-ribosylation of EF2 in cell extract was assayed as described above. Inhibition of protein synthesis was measured by incorporation of 3 H-leucine 15 hrs after toxin exposure, and cell death was assessed by MTS assays (see FIG. 2).

The IC_{50} is the toxin concentration that reduces protein synthesis by 50% compared to untreated cells and LC_{50} is the concentration that kills 50% of the cells (as measured in MTS assays). The number of TNF receptors per cell and affinity was determined by ^{125}I -TNF alpha (Amersham) competition and displacement assays using the program "Ligand" for data processing (Munson, P.J., and Rodbard, D., Meth. Enzymol. 92: 543-576, 1983). The mean of those
5 experiments \pm S.E. is shown; within this error range, displacement assays gave slightly lower apparent receptor number and higher affinity than the mean and competition assays lower affinity and higher receptor numbers.

Thus, the mechanism by which the cells containing CAS antisense become resistant is "downstream" of the primary damage done to the cells by the toxin. One explanation for the observed protective effects of the CAS antisense plasmid on cells is interference with expression of the corresponding cellular gene. To evaluate this possibility, we
10 examined the levels of CAS protein in MCF-7 cells containing the CAS antisense clone (pCDM/HE17; labeled MCF-7/17 on FIG. 3), containing the CAS sense clone (pCDM/HE17R; labeled MCF-7/R on FIG. 3) and control cells without a CAS clone (labeled MCF-7/N on FIG. 3). Using standard Western immunoblotting techniques (Sambrook, J., et al., Molecular cloning. A Laboratory Manual, 2nd ed., 1989) and CAS-specific polyclonal antibodies (see Example 13 for details on antibody preparation), we found equal amounts of a CAS protein (of expected size about 100 kDa) in MCF-7/N and MCF-
15 7/R cells, and reduced amounts in MCF-7/17 cells. FIG. 3 shows that CAS protein in cells containing the CAS antisense clone was detected in reduced amounts equal to about $65\% \pm 15\%$ of that detected in the control MCF-7 cells. This result shows that the presense of CAS antisense reduces the intracellular levels of the CAS gene product.

EXAMPLE 4: GROWTH OF TOXIN TREATED CELLS MADE RESISTANT TO TOXIN-INDUCED APOPTOSIS BY ANTISENSE CAS EXPRESSION

20 To gain more information about the status of the cells that are resistant to PE, time course experiments were carried out at two concentrations of PE (10 and 30 ng/ml). These concentrations were chosen because they had maximal inhibitory effect on the growth of control cells (see FIG. 2A) and maximally inhibited protein synthesis (see FIG. 2C). In addition, cells containing sense plasmids as well as other controls were included. Figure 2D-2F show the time course of treatment with PE. The MCF-7/17 cells containing CAS antisense were treated with 10 ng/ml of PE for three days
25 (see FIG. 2E) and showed little or no growth compared to untreated cells (compare to FIG. 2D). However, when the PE was removed the cells began to grow and by day 8 reached almost the same cell number as the control cells that were not treated with PE. Figure 2F shows that MCF-7/17 cells that were treated with 30 ng/ml of PE for 3 days showed no growth, but after removal of PE, recovered and resumed growth. In contrast, MCF-7/N cells, which contained only the pMC1neo/polyA plasmid, and MCF-7/17R cells which contained the pMC1neo/polyA plasmid and the pCDM/HE17R
30 plasmid (CAS sense), showed no growth in the presence of 10 or 30 ng/ml of PE and did not recover after toxin removal (Figs. 2E and 2F). This phenotype was also observed with the other control cell lines plasmids MCF-7/C and MCF-7/C2-C5.

These results show that cells containing the CAS antisense plasmid remain alive and begin to proliferate when the toxin is removed, in contrast to control cells which die as a consequence of toxin treatment. The results also show
35 that the effect of the antisense plasmid is specific because cells containing the sense plasmid (MCF-7/17R), as well as other control plasmids, did not recover after toxin removal.

The morphology of cells exposed to toxins reflects the changes observed using the MTS and ^3H -leucine incorporation assays. Cells were exposed to PE and TNF and the morphology of the cells was observed by phase contrast microscopy of a random field (at 250 x magnification) after incubation with PE (10 ng/ml) or TNF alpha (1 ng/ml) for 3 days. Control cells (MCF-7/N) become rounded, refractile and detach from the plate surface indicating cellular death. The MCF-7/17 cells transfected with the CAS antisense plasmid remained attached to the plate surface.

The morphologic appearance of MCF-7/N cells and MCF-7/17 cells (containing the antisense plasmid) was observed without toxin treatment and after treatment with 10 ng/ml of PE for two days. The untreated cells grew as flattened cells in islands characteristic of MCF-7 cells. There was no obvious morphological difference between the MCF-7/N cells and MCF-7/17 cells. However, the appearance of the cells treated with 10 ng/ml of PE for two days was very different. No MCF-7/N cells were detected attached to the dish; all the cells were floating, refractile and many were disintegrating. In contrast, most of the MCF-7/17 cells remained flattened and attached to the dish and resembled cells that had not been treated with PE. This morphology occurred under conditions where the cells' ability to incorporate ^3H -leucine into protein was arrested to background levels (see FIG. 2C at 10 ng/ml PE). This normal morphology was consistent with the cells' ability to resume growth when the toxin was removed (see FIG. 2E and 2F).

15 EXAMPLE 5: DNA DEGRADATION IS DECREASED IN CELLS MADE RESISTANT TO TOXIN-INDUCED APOPTOSIS BY ANTISENSE CAS EXPRESSION

Some cells undergo apoptosis upon exposure to ADP-ribosylating toxins (Kochi, S.K., and Collier, R.J., *Exp. Cell Res.* 208: 296-302, 1993; Chang, M.P., et al., *J. Biol. Chem.* 264: 15261-15267, 1989; Morimoto, H., and Bonavida, B., *J. Immunol.* 149: 2089-2094, 1992). Because MCF-7 cells containing the CAS antisense plasmid become less sensitive to ADP-ribosylating toxins without changing their susceptibility to toxin-mediated protein synthesis inhibition, it is likely that the CAS antisense affects the susceptibility of cells to undergo apoptosis. One hallmark of apoptosis is nuclear DNA degradation and the formation of a DNA ladder due to internucleosomal cleavage of chromosomal DNA (Compton, M.M., *Cancer Metast. Rev.* 11: 105-119, 1992). Therefore, cells treated with PE were used as a source of DNA (from the medium and soluble cell fraction) which was analyzed as described by Kochi and Collier (*Exp. Cell Res.* 208: 296-302, 1993). Release of DNA fragments into cell culture supernatant was analyzed by labeling cells for 20 hrs with ^3H -thymidine (Amersham), then changing the medium and exposing cells to toxin as described (Kochi, S.K., and Collier, R.J., *Exp. Cell Res.* 208: 296-302, 1993). DNA release was assayed by measuring radioactivity in the medium and in the soluble fraction of cells and comparing that to the total radioactivity.

Figure 4 shows that CAS antisense renders MCF-7 cells less susceptible to apoptosis as determined by nuclear DNA degradation. Toxin treatment induced degradation of chromosomal DNA as determined by two assays: radioactive measurement of degraded DNA present in the culture medium and visualization of the degraded DNA following gel separation.

To quantitate DNA degradation, 10^6 cells were labeled with ^3H -thymidine (1 mCi/ml) overnight and then treated with various amounts of PE for 15 hrs. The amount of ^3H -thymidine in chromosomal DNA, the cellular soluble fraction and the medium was determined by scintillation counting as described (Kochi, S.K., and Collier, R.J., *Exp. Cell Res.* 208: 296-302, 1993).

As shown in FIG. 4, with increasing concentrations of PE, there is a concomitant increase in DNA released into the medium and into the soluble fraction of cells. Because intact genomic DNA is usually not present in the culture medium and the soluble cell fraction of healthy cells, this indicates extensive DNA degradation upon toxin treatment of control cells. In contrast, in MCF-7/17 cells containing CAS antisense, the production of soluble DNA was greatly diminished at all PE concentrations tested (shown in FIG. 4).

The nature of the soluble DNA was determined by isolating DNA from the medium, which contained most of the soluble DNA, and subjecting it to electrophoresis and autoradiography to detect the sizes of soluble DNA (i.e., to detect a "ladder" characteristic of internucleosomal DNA degradation). When analyzing internucleosomal DNA degradation we found that DNA of cells still attached to culture dishes was in most cases a smear (50,000-200 bp) and the ladder fragments were difficult to visualize. However, DNA obtained from cell culture fluid by phenol/chloroform extraction and ethanol precipitation clearly showed a ladder. Therefore, DNA obtained from the medium was used to determine the extent of nuclear degradation and the sizes of the degraded DNA fragments. ³H-labeled DNA was isolated from the medium by phenol extraction and ethanol precipitation and separated by electrophoresis on a 1.5% agarose gel which was used to produce an autoradiograph.

The PE treated MCF-7/N cells displayed a typical nucleosomal ladder having wide bands of about 150-225 bp, about 300-425 bp, about 450-600 bp and in additional steps up to about 1500 bp. In contrast, the MCF-7/17 cells showed mostly higher molecular weight fragments (greater than 1500 bp) and a very faint DNA ladder of similar sizes as seen in the MCF-7/N cells but that was so faint it could not be photographically reproduced. Our finding that DNA degradation and nucleosomal ladder formation is diminished in MCF-7/17 cells containing CAS antisense suggests that toxin resistance is due to interference with an apoptosis pathway. **EXAMPLE 6: EXPRESSION OF ANTISENSE CAS MAKES CELLS LESS SUSCEPTIBLE TO APOPTOSIS INDUCED BY TUMOR NECROSIS FACTORS ALPHA AND BETA**

It is well established that apoptosis can be induced in certain cell lines by TNF alpha and TNF beta. The toxins PE and DT produce cell death by causing inhibition of protein synthesis by modification of EF2, whereas TNF acts by receptor a different mechanism involving binding and signal transduction (Smith, C.A., et al., *Cell* 76: 959-962, 1994). Although two different mechanisms cause cell death, it has been suggested that DT-mediated and TNF-mediated cytotoxicity might share a common pathway leading to cell death (Morimoto, H., and Bonavida, B., *J. Immunol.* 149: 2089-2094, 1992). Thus, if this pathway were part of the apoptosis machinery, one might expect that cells that are resistant to PE-induced apoptosis might also be more resistant to TNF-induced apoptosis.

TNF binding was first demonstrated to show that MCF-7 cells express TNF receptors. Binding assays were performed essentially as previously described (Webber, K.O., et al., *Mol. Immunol. in press*, 1995). 3×10^5 cells per dish in 24-well plates were chilled and blocked with RPMI/5% BSA/50 mM BES/50 mM BES pH 7 for 1 hr at 4°C and washed twice with binding buffer (RPMI/1% BSA/50 mM BES, pH7). ¹²⁵I-labeled TNF alpha and cold TNF in binding buffer were then added and incubated for 2 hrs. For competition experiments, 80 pM final conc. of ¹²⁵I-TNF, 12 nCi/16 fmol/well (Amersham) was mixed with varying concentrations of unlabeled TNF. For Scatchard experiments serial dilutions, 18 to 70 pM, of labeled TNF were used. Labeled TNF was then removed and the cells were washed twice

with binding buffer. Cell bound radioactivity was recovered in 0.5% SDS in TE and determined in a Beckman 5500B gamma counter. Receptor numbers and affinity was calculated using the LIGAND program (Munson, P.J., and Rodbard, D., *Meth. Enzymol.* 92: 543-576, 1983). Table 1 and Figure 5 show that MCF-7 cells have TNF receptors and are sensitive to TNF mediated apoptosis.

5 The effects of CAS antisense plasmids on TNF induced apoptosis in MCF-7 cells was then determined. ³H-leucine incorporation assays were used to quantitate killing by TNF. The assays were performed 3 days after TNF alpha (FIG. 5A and 5B) and TNF beta (FIG. 5C). FIG. 5 shows that CAS antisense reduces the sensitivity of MCF-7 cells to TNF. The IC₅₀ of TNF alpha on untransfected MCF-7 cells and cells containing control plasmids was approximately 0.1-0.2 ng/ml (FIG. 5A and 5B). The IC₅₀ of TNF beta was 2-3 ng/ml for the control (FIG. 5C). In contrast, MCF-7/17 cells
10 bearing the CAS antisense plasmid were about 5-10-fold less sensitive to both TNF alpha (IC₅₀ about 1 ng/ml) and to TNF beta (IC₅₀ about 20 ng/ml) (FIG. 5A-5C).

This difference in TNF sensitivity between control cells and CAS antisense cells was also seen by examining the morphological appearance of TNF treated cells under phase contrast microscopy. Control cells treated with 1 ng/ml TNF were detached from the dishes and were disintegrating, while CAS antisense cells stayed attached to the dishes
15 and resembled untreated cells. This phenotype was very similar to the effects seen with PE treated cells as described in Example 4.

Time course experiments were used to determine cell viability by MTS assays in 96 well plates as described in Examples 1 and 2. Similar to the phenotype of CAS antisense transfected MCF-7 cells that were treated with PE, TNF treatment of these cells produced growth inhibition, which was reversed after TNF removal (see FIG. 5D for TNF
20 alpha and FIG. 5E for TNF beta, and compare to FIG. 2D-F). An average growth curve (mean of three experiments) of cells without toxin is indicated by a broken line and the duration of TNF exposure is indicated by a bar on the X-axis. MCF-7/17 and MCF-7/17Δ contain CAS antisense. Controls were MCF-7/17R that contains the sense plasmid, MCF-7/C that contains the pCDM8 library vector without insert, and MCF-7/C2 that contains a randomly chosen library plasmid with an unknown insert (see FIG. 1B for comparison). The control cells did not survive TNF treatment and did not
25 recover after TNF removal.

The altered sensitivity of cells to TNF when the CAS antisense plasmid was present was not due to interference with the immediate action of TNF, which is binding to its receptor. Figure 5F and Table 1 show that the TNF receptor numbers and affinity remained unchanged in MCF-7/17 cells containing CAS antisense.

EXAMPLE 7: THE DNA SEQUENCE OF A COMPLETE CAS cDNA REVEALS HOMOLGY TO THE YEAST 30 CHROMOSOME SEGREGATION GENE CSEI

The CAS antisense plasmid that reduces the sensitivity of cells to PE, DT and TNF contained only a 436 bp fragment of a CAS cDNA. To obtain a complete (normal human) CAS coding sequence, we used the cDNA insert of pCDM/HE17Δ as a probe to screen a human placenta cDNA library in lambda gt11.

1 x 10⁶ plaques of a Lambda gt11 cDNA library from placenta polyA RNA (Clontech™) were screened by
35 hybridization with a radioactivity labeled cDNA insert of pCDM/HE17Δ using standard techniques (Sambrook, J., et al,

Molecular cloning. A Laboratory Manual., 2nd ed., 1989). The largest inserts, about 2.4 kb, included the 3'-end but not the 5'-end of the cDNA with an estimated size of 3.2 kb.

Although several cDNA inserts were isolated, the longest being about 2.4 kb, no full length clone was obtained. A full length clone was expected to be about 3.2 kb from the mRNA size in Northern blots (discussed in detail in Example 5 8 and shown in FIG. 7). This size was also expected if the CAS cDNA were about the same size as the yeast CSE1 cDNA.

Because all of the isolated clones were from the 3' end of the cDNA, the 5' end of the cDNA was obtained by the RACE method (Apte and Siebert, *Biotechniques* 15: 890-93, 1993; Frohman et al., *Proc. Natl. Acad. Sci. USA* 85: 8998-9002, 1988) using 5'-RACE-Ready™ placenta cDNA (Clontech) as template and the Clontech anchor primer 5'-
10 CTGGTTCGGCCACCTCTGAAGGTTCCAGAATCGATAG-3' (SEQ ID NO:3) and a CAS specific primer 5'-
TAATGAGGTCTCTCAGAAA-3'(SEQ ID NO:4) positioned 160 bp downstream of the 5' end of the longest lambda insert. Amplification was done for 30 cycles (2 min at 94°C, 2 min at 60°C, 2 min at 72°C and a final extension of 10 min at 72°C) using a Perkin Elmer GeneAmp XL™ PCR kit. A single 1160 bp fragment was obtained which was the correct 5' end of the CAS because the overlap of this fragment 3' end matched with the first 160 bp of the longest previously
15 obtained lambda clone 5' end. The lambda fragments and RACE fragments were cloned into pCRII (Invitrogen) and sequenced with an ABI™ 373A sequencer and ABI Dye-Deoxy Terminator™ kit.

The nucleotide sequence of the cDNA clone is SEQ ID NO:1 (GenBank acces. no. U33286), and deduced amino acid sequence of the CAS protein is SEQ ID NO:2, as shown in FIG. 6A and FIG. 6B. The CAS cDNA from HeLa used as a screening probe had the same sequence as the human placenta cDNA and corresponds to nucleotides 2100-2536
20 of the full-length clone. The coding regions of human CAS and yeast CSE1 (Xiao, Z., et al., *Mol. Cell. Biol.* 13: 4691-4702, 1993) are of approximately the same size, 971 and 960 amino acids respectively, and their sequence is similar over their whole length with some small gaps, resulting in overall homology of about 59%. The deduced translation products have approximately the same size and are homologous as determined by performing homology analysis and motif searches using the Genetics Computer Group package version 8 (Madison, WI) or the BLAST (Altschul, S.F. et al., *J. Mol.*
25 *Biol.* 215:403-410, 1990) analysis with the National Center for Biotechnology Information network service. The overall homology (protein similarity) is 59% and in some portions, the homology is greater than 75% with about 50% identity. For example, CAS protein sequence showed identity with the CSE1 protein sequence over amino acid stretches of 11 amino acids (in one region), 9 amino acids (in one region), 8 amino acids (in one region), 7 amino acids (in one region), 8 amino acids (in two regions) and regions of 5 or fewer identical amino acids in the aligned sequences. No defined
30 protein other than CSE1 showed significant homology to CAS. However, some previously sequenced but functionally undefined short expression sequence tags ("EST" sequences) of between 209 and 346 bp were found to have homology or to match identically to the 5' or 3' end of CAS cDNA. There was no significant protein sequence motif in the CAS sequence that would indicate its molecular function although some short sequence stretches are similar in motif to phosphorylation sites of protein kinases ERK1 and ERK2 or the transcription factor TFIID, to basic regions (amino acids
35 372-385) that may be involved in nuclear localization or DNA binding, and to zinc-finger proteins (amino acids 613-630).

The homology of the CAS gene relative to the yeast CSE1 gene shows that these are conserved genes and therefore the proteins encoded by these genes are likely to have similar important cellular functions. For comparison, the yeast CSE1 is an essential gene involved in cell division (i.e., homozygous CSE1 mutations are lethal; Xiao, Z., et al., *Mol. Cell. Biol.* 13: 4691-4702, 1993). However, the function of the human CAS gene in the process of apoptosis, as defined by the results in Examples 3-6, differs from that described for the CSE1 gene in yeast.

EXAMPLE 8: EXPRESSION OF CAS IN NORMAL HUMAN TISSUES AND HUMAN CANCER CELL LINES

To determine if CAS may have a role in cell proliferation as well as apoptosis, the expression of CAS in human tissues and in tumor cell lines was analyzed by Northern blotting (Sambrook, J., et al., Molecular cloning. A Laboratory Manual, 2nd ed., 1989). Northern blots containing about 2 μ g polyA-containing mRNA of various tissues separated on a denaturing formaldehyde 1.2% agarose gel (Clontech) were hybridized with radioactive labeled CAS probe (insert of pCDM/HE17 Δ ; 32 P random primed, about 10^9 cpm/ μ g) or actin and/or GAPDH control probes (Clontech) having comparable specific activities for 20 hrs at 50°C in Hybrisol 1™ (50% Formamide, Oncor) solution. Blots were exposed to Kodak XAR2™ film (-70°C with screen) for 18-20 hrs (to detect CAS) or about 6 hr (to detect actin and GAPDH), and then scanned on a Molecular Dynamics Phosphorimager 425™. RNA levels were quantitated by comparing the signals of CAS to the actin and/or GAPDH signals.

The control hybridizations showed that approximately equal amounts of RNA were loaded for most tissues except for skeletal muscle which could not be exactly quantitated due to additional actin band and apparently elevated GAPDH. Some tissues were tested in duplicate to demonstrate the reproducibility of the results.

The CAS mRNA, detected band of about 3kb, was found in many tissues including pancreas, adrenal medulla, thyroid, adrenal cortex, testis, thymus, small intestine, stomach, spleen, lymph node, thymus, appendix, peripheral blood, bone marrow, fetal liver, spleen, prostate, ovary, colon, heart, brain, placenta, lung, liver, skeletal muscle and kidney. A quantitation of CAS expression, normalized to the expression of actin or GAPDH is shown in FIG. 7. Relative mRNA levels in tissues and tumor cell lines were determined by comparing the CAS intensities with the mean of actin and GAPDH signals using a Molecular Dynamics Phosphorimager™. For determining "relative expression" the CAS/Control ratio found in most tissues (the mean of levels found in prostate, small intestine, colon, heart, brain, lung and pancreas) was defined as a "basal level" and were set to 1. Testis, which is the tissue having the highest expression level, was set to 100. The others were determined relative to these tissues.

Expression was very high in testis and fetal liver which contain many actively proliferating cells, and was elevated (above basal level) in tissues that contain some proliferating cells, e.g. lymphoid tissues. Expression was slightly elevated in skeletal muscle. Very low expression was detected in peripheral blood which does not contain proliferating cells.

In addition, various proliferating tumor cell lines, e.g. SW480 (colon), A549 (lung) and HL60, K562, Molt4 and Raji (leukemias and lymphomas) contained high levels of CAS mRNA.

Based on these results, expression of CAS can be used as a marker for cell proliferation. Because cell proliferation is a hallmark of cancer cells, detecting CAS expression in cells above basal levels can serve as a method for detecting cancer cells.

EXAMPLE 9: THE CAS GENE IS LOCATED IN THE 20q13 REGION OF HUMAN CHROMOSOME 20

To identify the chromosomal localization of the CAS gene, Southern blot hybridization of a CAS probe to a panel of somatic cell hybrids that represent the different human chromosomes in a mouse or hamster background was performed. Under stringent conditions, a 436 bp cDNA fragment covering the CAS cDNA from position 2100 to 2536
5 (see FIG. 6A) was hybridized to the panel.

A southern blot containing human (male and female) genomic DNA cut with PstI, mouse and hamster DNA, and genomic DNA from somatic cell hybrids containing single human chromosomes was obtained from ONCOR and hybridized under stringent conditions with the CAS probe. On the autoradiograph, mouse and hamster lines were easily distinguished because under these stringent conditions, hybridization of CAS to mouse DNA was easily seen, but no hybridization was
10 detected to hamster DNA. The CAS probe hybridized to human female and male DNA and to DNA from the mouse cell line that carries a human chromosome 20.

Two PstI fragments of human genomic DNA specifically hybridized with the CAS probe as expected because the probe contained an internal PstI site. Thus at least two fragments would be expected when genomic CAS DNA is cut with PstI. Of the different somatic cell hybrids containing one human chromosome each (in mouse or hamster cell
15 background), these two fragments were detected in the cell line that contained human chromosome 20. Thus, based on these hybridization results, the CAS gene is located on chromosome 20.

To further map the CAS gene, we localized the gene on human DNA contained in yeast artificial chromosomes (YAC). The Centre d'Etude du Polymorphisme Humaine (CEPH) human YAC Megabase library (Cohen, D. et al. *Nature* 366:698-701, 1993) was screened by PCR using the primers P1 (5'GACATCCCGTCTTCCTATATG) (SEQ ID NO:5) and
20 P2 (5'AAGAAAGCCTCACTAGAGCAGGA) (SEQ ID NO:6) which bound to and amplified a 90 bp fragment when CAS cDNA and human genomic DNA were individually used as templates. One YAC clone, 953-B-4, was specifically amplified with these primers.

Microsatellite markers were identified on or near the YAC 953-B-4 address using techniques well known in the art and this information was used to position the CAS gene relative to typed microsatellite markers in the YAC library.
25 YAC 953-B-4 overlaps with YACs positive for the genetic marker D20S176, which is mapped to the long arm of human chromosome 20, indicating that the CAS gene is located within a 2-3 Mb region of the genetic marker D20S176 on chromosome 20.

This position was confirmed by Fluorescent In Situ Hybridization (FISH; described in greater detail below) (Thompson, C.T. & Gray J.W., *J Cell. Biochem. (Suppl.)* 176: 139-143, 1993) analysis using a P1 clone containing the
30 human CAS gene as probe (see Example 10). This position is close to the 20q13 region that is amplified in certain breast tumors and breast cancer cell lines (e.g., HTB20), and in colon and bladder cancer cell lines. This region is known to contain amplifications that correlate with aggressive progression of breast cancer and probably harbors one or more oncogene(s) responsible for the aggressive cancer phenotype (Tanner M.M. et al., *Cancer Res.* 54, 4257-4260, 1994).

EXAMPLE 10: THE CAS GENE IS AMPLIFIED IN HUMAN CANCER CELL LINES

35 Because the CAS gene is located in a region known to undergo amplification in tumor cells, the amplification of the CAS gene was examined in a number of tumor cell lines using Southern blotting.

Quantitative Southern hybridizations were used to determine the relative number of copies of the CAS gene in genomic DNA of tumor cell lines using techniques well known to those skilled in the art (Sambrook, J., et al., Molecular cloning. A Laboratory Manual, 2nd ed., 1989). Genomic DNA from a variety of cancer cell lines including breast, colon, bladder, gastric, ovary, prostate, melanoma, leukemia and lymphoma were tested. As shown in FIG. 7, the cell lines analyzed were BT-474 (also known as HTB20), MCF-7, LS174T, COLO205, SW-480 and N-87 (all of which are available from the American Type Culture Collection, Rockville, MD). As an internal control, the actin gene was also hybridized to determine the normal gene copy number without amplification. This probe is an appropriate control also for cell lines which are described to be aneuploid, because chromosome 7, which harbors the actin gene, is over-represented in breast cancer cell lines (HTB20). Therefore, this control probe gives a very conservative estimate of CAS amplification (because it can already be elevated itself) and can compensate potential over-representation of chromosome 20 in aneuploid cell lines.

FIG. 8 shows the comparison of the ratio of the hybridization signals between control signal and CAS signal in different cell lines hybridized with the CAS probe and actin control probes. The ratio between CAS and actin control signals was determined by quantitatively scanning the bound radioactivity on the Southern blots using a Phosphorimager™. Human genomic DNA was used as a reference for single copy status of CAS (the actin/CAS signal ratio was set to 1 for human genomic DNA).

The ratio for normal human genomic DNA was set to 1 and the other samples are relative to that. If one assumes a linear relation between gene targets on the filter and hybridization signal, this ratio approximates the copy number of CAS. The results shown in FIG. 8A show that the CAS gene is amplified in the breast cancer cell line BT-474 (HTB20), and colon cancer cell lines COLO205 and SW-480 when compared to the normal human genomic DNA controls and to cancer cell lines MCF-7 (breast cancer), LS174T (colon cancer) and N-87. FIG. 8A shows the results for two breast cancer cell lines and FIG. 8B shows the results for colon cancer cell lines (SW480, COLO205, LS147T) and a gastric cancer (N87) cell line. The CAS gene was also amplified about two-fold in the colon cancer line COLO201 and the leukemia cell line CEM, although there was no amplification of CAS found in three other leukemia cell lines (K562, HL60 and KG1) (data not shown).

The CAS gene was amplified in the breast cancer cell line HTB20 which was previously described to contain a 20q13 amplification. In contrast, the CAS gene was not amplified in a cell line (MCF-7) that does not contain the described amplification.

The 20q13 region that is found to be amplified in certain cancers and cell lines is not a homogenous region but consists of different regions that lie close together and contain various degrees of (possibly independent) amplifications. For example, Tanner et al. (*Cancer Res.* 54, 4257-4260, 1994) defined one minimal high amplification region, but also noted surrounding regions being amplified to a lesser degree, and different amplifications in different breast cancer cell lines. Although a close link of the CAS gene to the minimal amplification region in 20q13 has been established by the YAC localization (see Example 9), more detailed information about CAS amplification in cancer cells was obtained by FISH analysis (Thompson, C.T. & Gray J.W., *J Cell Biochem.* (Suppl) 17G: 139-143, 1993) using the

P1/hCAS clone (see Example 11) on BT474 breast cancer cells which showed CAS amplification in quantitative Southern hybridizations.

Interphase and metaphase chromosomes were hybridized with digoxigenin-dUTP-labeled P1/CAS and biotin-dUTP-labeled control probes P1/2672 and P1/2567 (Genome Systems; located close to CAS on 20q) or P1/5461 (Genome Systems; located on the 20p arm). The control clones P1/2672 and P1/2567 were mapped by Genome Systems previously at about 2 Mb centromeric (for P1/2672) and telomeric (for P1/2567) from CAS on chromosome 20 or on 20p (for P1/5461). P1/CAS was visualized by fluorescein-anti-digoxigenin antibody (green) and the controls with Texas Red-avidin (red), using a triple band pass filter set and DAPI counterstain.

FISH analysis showed that CAS was amplified 74 fold (up to 14 copies per cell) in these cells and also that the CAS gene sequence was translocated from the 20q13 locus to various other unidentified chromosomal locations.

To analyze whether CAS is amplified in other cancer cell lines in addition to the breast cancer line, we analyzed by Southern blot hybridization genomic DNA from three cell lines derived from colon cancer (COLO205, LS174T and SW480) and one gastric cancer cell line (N87). Again, human genomic DNA was used as a single copy CAS reference control. Figure 8B shows that DNAs of the colon cancer cell lines SW480 and COLO205 had stronger CAS signals than human genomic DNA. The other cell lines showed approximately the same CAS signal. The quantitation of the Southern hybridization signals by Phosphorimager™ analysis indicated a 2-fold amplification of CAS DNA in the colon cancer cell lines COLO205 and SW480. Thus, CAS is amplified not only in cell lines of breast cancer but also in other types of cancer including two out of three colon cancer cell lines that were analyzed. FISH analysis showed that this amplification in colon cancer cells was not due to specific amplification of CAS DNA alone. Instead, in these cells, the entire chromosome 20 or a larger portion of the chromosome (e.g., the 20q arm) was amplified.

P1/CAS contains most (and probably all) of the CAS gene because: (1) PCR primers for the 5' portion and 3' portion of the cDNA amplify 5' and 3' CAS gene fragments when using P1/CAS as template; (2) 5'- and 3'-end cDNA probes hybridize to P1/CAS; and (3) DNA sequencing reveals the presence of the CAS coding region in that phage. P1/CAS was labeled with digoxigenin and hybridized to interphase and metaphase chromosomes and detected with fluorescein-labeled anti-digoxigenin antibodies. As reference probes for the determination of the relative amplification status of CAS, we used the following biotin-labeled P1 clones: P1/2672, which hybridizes on 20q, centromeric to the position of the CAS gene in 20q13; P1/2567, which is telomeric to CAS; and P1/5461, which maps to the p arm of chromosome 20. The normal cell control (peripheral blood lymphocytes or PBL) showed two copies with each probe on the long arm of chromosome 20. Metaphase chromosomes showed a linear orientation with P1/2672 located centromeric and P1/2567 located telomeric to P1/CAS. These results confirm the position of P1/CAS on 20q13 as defined by YAC mapping.

CAS in Breast Cancer Cells. We then analyzed five breast cancer cell lines: BT-474, MDA-MB-157, UACC-812, MDA-MB-361, and MDA-MB-134. BT-474 was chosen because Southern analyses indicated potential CAS amplification. The others were evaluated because 20q13 amplifications occur frequently in breast cancer and because such amplifications were already demonstrated by comparative genomic hybridization (CGH) and FISH with other probes in lines UACC-812, MDA-MB-157, and BT-474 (Tanner et al, *Cancer Res.* 54:4257-4260, 1994). On BT-474 cells, pairwise hybridization showed 8 to 16 signals with P1/CAS but only two signals per cell with the centromeric control

probe. The two centromeric signals were linked to P1/CAS in a chromosome that appears to be the remainder of chromosome 20. Thus, relative to the region centromeric of CAS, as defined by the control phage (P1/2672), the CAS gene is amplified four- to eight-fold. Pair-wise FISH on BT-474 cells with P1/CAS and P1/2567 (as a control that hybridizes telomeric of CAS) showed 8 to 12 signals (in one experiment, up to 18) with CAS and 4 signals with P1/2567.

- 5 Two of the four control signals were linked to CAS; the others were found on another chromosome separated from CAS. BT-474 cells do not contain a normal chromosome 20 and all three probes hybridized to morphologically abnormal chromosomes. The amplified CAS gene was translocated to different abnormal chromosomes, each containing one to five copies of CAS. No double minute chromosomes were seen in BT-474 cells.

The other breast cancer cell lines that we analyzed, MDA-MB-134, MDA-MB-157, MDA-MB-361 and UACC-812
10 contained three to six copies of CAS, mainly present on abnormal marker chromosomes. MDA-MB-361 contained two normal copies of chromosomes 20 (with CAS) and one additional copy of CAS separated from the telomeric 20q control on an aberrant chromosome. These cells also had a third copy of the control gene separated from CAS on another abnormal chromosome. MDA-MB-134 cells contained four to five copies of CAS, two to three normal copies of chromosome 20, and two CAS genes on a 20q isochromosome. UACC-812 and MDA-MB-157 contained three to six
15 copies of CAS and of the centromeric control. Both signals were linked on abnormal chromosomes. UACC-812 cells contain abnormal chromosome 20 material with the centromeric control probe amplified to a higher degree than CAS (to five to eight copies), consistent with the observation of a second amplified region centromeric from CAS in this cell line (Tanner et al., *Cancer Res.* 54:4257-4260, 1994).

CAS in Colon Cancer and Leukemia Cells. Three colon cancer cell lines (SW480, COLO201 and COLO205)
20 were identified as amplification candidates by Southern analyses and were analyzed by FISH with P1/CAS, the control probes described above, and another 20p probe (P1/5461). In SW480 and COLO205, CAS was located on aberrant chromosomes containing parts of 20q, similar to MDA-MB-157 and UACC-812 but in contrast to BT-474 cells. The extra CAS copies were linked to 20q controls but separated from 20p. The elevated copy number that was apparent in Southern blots of COLO201 (colon and CEM (leukemia) cells is attributable to extras copies of chromosome 20. We
25 found five copies of chromosome 20 per COLO201 cell and six to eight copies per CEM cell.

The results of these studies on CAS gene amplification in selected cancer cell lines are summarized in Table
2. The relative numbers of the CAS gene were determined compared to normal human peripheral blood lymphocytes (PBL) which have no amplification of the CAS gene (i.e., two copies of the CAS gene by FISH analysis in the 46 chromosomes per cell). All of the cell lines shown in Table 2 have some amplification of the CAS gene, often associated with some
30 chromosomal abnormalities, such as an extra copy of chromosome 20 ("extra 20"), an aberrant chromosome 20 ("aberrant 20") such as no normal chromosome 20 but detection of 20q marker chromosomes, an amplification of the 20q region ("large 20q"), or a CAS-specific amplification and translocation to another chromosomal region than 20q ("CAS translocation").

Table 2: CAS Gene Amplification in Selected Cancer Cell Lines

Cell Line	Cancer Type	CAS Copies (FISH)	Chromosomes per cell	Fold Amplification	Observations
MDA-MB-134	Breast	4	86	< 2	
MDA-MB-157	Breast	2-6	54	< 3	large 20q
UACC-812	Breast	3	60	< 2	aberrant 20
MDA-MB-361	Breast	3	56	< 2	aberrant 20
BT-474	Breast	8-16	95-100	3-8	aberrant 20; CAS translocation
COLO201	Colon	5	57-60	≈ 2	extra 20
COLO205	Colon	5	65-69	≈ 2	extra 20; large 20q
SW480	Colon	4-6	48-55	≈ 2	aberrant 20; large 20q
CEM	Leukemia	6-8	83-86	≈ 2	extra 20

15

FIG. 9 shows the relative location of the CAS gene on 20q13 relative to other markers on an ideogram adapted from Tanner et al. (*Cancer Res.* 54:4257-4260, 1994). The other markers are "GHRH" for growth hormone releasing factor; "RPN2" for riboprin 2; "SRC" for oncogene *src*; "TOP1" for topoisomerase 1; "ADA" for adenosine deaminase; "SEMG1" for seminogelin 1; "PTPN1" for protein tyrosine phosphatase; "MC3R" for melanocortin-3 receptor; "RMC20C001" for Tanner et al.'s amplification probe; "PCK1" for PEP carboxykinase; "GNAS" for guanosine NT-binding protein; "CHRNA4" for cholergeric receptor α -4; "EDN3" for endothelin 3; and "ZNF8" for zinc finger protein 8.

Because CAS is like other known oncogenes related to apoptosis and cell proliferation, and it is amplified in cell lines of breast and other cancers, the CAS gene may plays a role in cancer. Furthermore, because amplification of the 20q13 region in which the CAS gene is located correlates with aggressive breast cancer, the detection of CAS amplification or other alternations involving the CAS gene are valuable as a predictive factor in cancer diagnosis and therapy.

EXAMPLE 11: ISOLATION OF GENOMIC CLONE CONTAINING THE HUMAN CAS GENE

A P1-clone containing the CAS gene, called P1/hCAS, was isolated from a human genomic P1-library by standard molecular biology and PCR techniques as described by Sternberg (*Trends in Genetics* 8:11-16, 1992). The PCR primers P1 comprising 5'GACATCCCCTCTTCCTATATG 3' (SEQ ID NO:5) and P2 comprising 5'AAGAAGCCTCACTAGAGCAGGA 5' 3' (SEQ ID NO:6) located in the human CAS cDNA, which amplify a 90 bp CAS-specific DNA fragment from human genomic DNA were used to identify a P1 clone from the P1-library of GENOME SYSTEMS that hybridized to these primers. This P1 clone was called P1/hCAS. Because the primers used bind specifically to the CAS gene DNA, the isolated P1-clone contains at least part of the human CAS gene.

The presence of the full length CAS gene in this P1 CAS clone was confirmed by PCR analysis of P1/hCAS 10 using the primer pair P51 comprising 5'TGTGAAGCCGATCGAGTGGC 3'(SEQ ID NO:7) and P52 comprising 5'TTCAGGGACATCCTGAAAGT 3' (SEQ ID NO:8) which amplify a 5'-end fragment from human CAS cDNA and P31 comprising 5'CCCCACTGATGGACACTGA 3' (SEQ ID NO:9) and P32 comprising 5' CTCACCATTGATGGAACCC 3' (SEQ ID NO:10) which amplify a 3'-end fragment from CAS cDNA (see FIG. 10 for a diagrammatic representation of these primers and their location on a hypothetical CAS gene). The rationale of using these primers and PCR analysis of 15 P1/hCAS to confirm the completeness of the gene is depicted in FIG. 10 which is a schematic diagram of the organization of typical eukaryotic genes and does not represent the actual arrangement of introns and exons in the CAS gene. P51 and P52 will amplify a DNA fragment from P1/hCAS only if the 5'-end of the gene is present. Similarly, P31 and P32 will only result in a PCR fragment if the gene portion containing the 3'-end of the CAS coding region is present. FIG. 10 shows these combinations of primers as bars above the box labelled "CAS mRNA (cDNA)".

20 Using this approach, a specific DNA fragment was obtained with the P51 and P52 primer combination in a PCR reaction when the P1/hCAS was used as the PCR template but not when the template was not included in the reaction. The DNA amplified using this combination of P51 and P52 primers was about 2000 bp long. The 3'-end was also present on the P1/hCAS clone because when the combination of primers P31 and P32 were used with the P1/hCAS clone as template a similar fragment was amplified by PCR, whereas no fragments was observed without the P1/hCAS 25 template.

A comparison of the fragment sizes obtained from this (3'-end) PCR with the distance of the primers in the CAS cDNA indicated that the 3'-end fragment is significantly larger than the distance on the cDNA. Thus, it is likely that an intron is present in between these primer positions in the human CAS gene.

30

EXAMPLE 12: PCR DETECTION OF CAS GENOMIC DNA

Cancer cells often contain genetic abnormalities relative to normal cells that can be detected at the genomic level. To detect such genetic abnormalities associated with deletions, translocations or amplification of the 20q13 region, a PCR-based assay using primers specific for the CAS gene sequence is used.

35 Using the P51 and P52 combination of primers (P51/P52), genomic DNA from tissue suspected of having cancerous cells is amplified by PCR for a limited number of cycles. Similarly, the same source of genomic DNA is used

as a template in a PCR reaction using the P31 and P32 combination (P31/P32) of primers (see also Example 11). As a control, genomic DNA from normal human tissue is similarly amplified in an independent reaction. The amplified DNA is then separated by gel electrophoresis, stained with a DNA intercalating dye, and the relative amount of DNA in each sample is determined by the degree of fluorescence of the amplified DNA under UV light. The amount of fluorescence in the amplification product from normal genomic DNA serves as a baseline control representing unamplified CAS sequences. In tissue in which the DNA containing the CAS gene is deleted (e.g., on one of the two chromosomes 20), a lesser amount of amplified CAS DNA is detected. In tissue in which the DNA containing the CAS gene is amplified, a greater amount of amplified CAS DNA is detected. In tissue in which the CAS gene has been translocated to another location, a lesser (or no) CAS gene amplification may be detected depending on the location of the translocation breakpoint, or fragments of different lengths may be obtained following PCR amplification.

For performing this PCR-based assay, the following methods are used. Genomic DNA is purified from human tissues suspected of containing cancerous cells and from normal tissue as a control using standard purification methods. All PCR reactions are done in a final volume of 50 μ l containing 250 ng of genomic DNA, 25 pmol of each primer (either the P31/P32 or the P51/P52 combination), 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl₂, 0.1% Triton X-100, 200 μ M of each dNTP and 2.5 units of *Taq*I DNA polymerase. Amplification is for 15 cycles each consisting of 5 sec at 92°C, 1 min at 65°C and 4 min at 72°C followed by a single cycle of 12 min at 72°C. After amplification, 20 μ l of each reaction is applied to an agarose gel for electrophoretic separation and staining using standard methods (Sambrook, J., et al., Molecular cloning. A Laboratory Manual, 2nd ed., 1989).

Using this procedure, DNA isolated from tissue taken from a breast tumor is examined and found to contain more CAS genetic material compared to DNA from normal breast tissue for both the P31/P32 and P51/P52 combinations of primers. Thus, amplification of the CAS gene is detected in the tumor tissue.

It will be appreciated by those skilled in the art that a kit including the P31/P32 and/or P51/P52 combinations of primers or other primers specific for PCR amplification of CAS gene sequences could be used to detect genetic amplification of the CAS sequence. Determination of appropriate combinations of primers is well known in the art and is easily done using the sequence provided in SEQ ID NO:1. Such PCR-based kits are useful for detection *in vitro* or *in vivo* of cancer cells in human tissues. In addition to the CAS-specific primers, such a PCR kit may include primers specific for other areas of the genome (particularly for Chromosome 20 sequences), reagents used in performing the PCR (including but not limited to water, salts, buffers, dNTP solutions, *Taq*I DNA polymerase or other heat-resistant DNA polymerases). The amplified DNA sequence could be detected by separation and visualization of the products using gel electrophoresis or by other methods well known to those skilled in the art including DNA hybridization, filter binding and other well known techniques for specifically detecting specific DNA molecules.

Furthermore, it will be appreciated by those skilled in the art that a PCR-based kit may also be used to detect increased expression of the CAS gene. For example, a kit that includes a primer for transcribing CAS mRNA into cDNA by use of a reverse transcriptase (or other enzymes capable of producing cDNA) and primer combinations for then PCR amplifying the cDNA so produced would detect increased CAS gene expression. The amplified DNA may be detected using any of the procedures discussed above or other DNA detection methods well known in the art.

Thus, kits for detection of CAS genetic material or expression of CAS genes would include the PCR primer combinations needed for amplification and one or more reagents for performing PCR. A PCR kit may also reagents for detecting the amplified DNA.

EXAMPLE 13: PRODUCTION OF ANTIBODIES THAT SPECIFICALLY RECOGNIZE CAS PROTEIN

- 5 To raise antibodies against the human CAS protein, we constructed plasmids for expression of fragments of CAS protein as recombinant His-tagged proteins in *E. coli* containing either amino acids 1-284 or 327-669 of CAS (Scherf, U. et al., *Proc. Natl. Acad. Sci. USA* 93:2670-2674, 1996).

Construction of Plasmids. DNA fragments encoding N-terminal or internal sequences of CAS were obtained by PCR using CAS cDNA as template. The primers 5'-GAGATCCTATACATATGGAAGTCCAGCGATG-3' (SEQ ID NO:11) 10 with ATG translation initiation codon as part of a *Nde* I site (underlined), and 5'-TATCGCTGGAATTCTCATCCTAC-3' (SEQ ID NO:12) with an *Eco*RI site (underlined) for fusion to a hexahistidine tag were used to amplify the sequence for the first 284 aa. For the internal CAS fragment (aa 327-669) we used the primer 5'-CAGTTTGTCAATATGCCTCATTATAAAATC-3' (SEQ ID NO:13) and 5'-GAAGCAAAGAATTCAGTGAAGACGTATG-3' (SEQ ID NO:14). *Nde* I (start codon) and *Eco*RI (His₆) sites are 15 underlined. PCR products were cloned into pCR II (Invitrogen, San Diego, CA). The inserts were isolated from these vectors as *Nde* I/*Eco*RI fragments and cloned into pET-23b(+) (Novagen). The resulting plasmids, pUS6 for expression of CAS₁₋₂₈₄ (clone C2.6) and pUS7 for CAS₃₂₇₋₆₆₉ (clone C46) were confirmed to be correct by DNA sequencing.

Expression and Purification of CAS Protein Fragments. Recombinant CAS fragments were produced in *E. coli* cells containing these plasmids and purified from solubilized inclusion bodies by Ni-affinity chromatography (on Quiagen 20 Ni-Chelation resin). The expression plasmids pUS6 and pUS7 were transformed into *Escherichia coli* BL21 (ΔDE3), expression induced at OD₆₀₀ of 2 with 2 mM isopropyl β-D-thiogalactoside, and the cells were harvested 2 h later. Both recombinant proteins accumulated in cytoplasmic inclusion bodies (IB), which were purified without preparation of spheroplasts prior to IB preparation. The IB were solubilized in 6 M GuHCl/0.1 M sodium phosphate/0.01 M Tris-HCl, pH 8.0/5 mM 2-mercaptoethanol, and 10 mg was loaded onto a Ni-NTA column (Qiagen). The column was washed with 25 50 ml of wash buffer (8 M urea/0.1 M sodium phosphate/0.01 M Tris-HCl/5 mM 2-mercaptoethanol) at pH 8.0, 20 ml of wash buffer at pH 6.3, and 20 ml of wash buffer at pH 5.9. The proteins were eluted with wash buffer at pH 4.5. Protein concentration was determined with Bradford assay reagent (Pierce) using bovine serum albumin (BSA) as a standard. After separation by SDS gel electrophoresis and Coomassie blue staining, only a single band of purified protein was detected for each of the clones corresponding to amino acids 1-284 or 327-669 of the CAS protein.

30 **Immunizations.** Rabbits were immunized with these purified proteins and polyclonal antibodies were produced that detected CAS protein on Western blots of total cell extracts from MCF-7 cells and W138 cells or from *E. coli* cells containing the recombinant CAS fragments. Each purified CAS protein fragment was diluted in phosphate-buffered saline (PBS), pH 7.4/1.5% Tween 20 (100 μg/ml or 180 μg/ml). Samples (250 μl) were diluted with 250 μl of Freund's adjuvant and 125 μl each was injected subcutaneously into four different sites of each HM(NZW)FBR rabbit (Hare- 35 Marland, Hewitt, NJ). Two rabbits were injected with each protein. Injection on day 1 was with complete Freund's

adjuvant, the booster injections on days 14 and 28 were with incomplete adjuvant. Test bleeds were obtained 7 days after each injection and in 2-week intervals after the last injection.

Antibody Purification. Antibodies from rabbit R2.6-I against the N-terminal portion (CAS₁₋₂₈₄) and from rabbit R46-I against the center portion (CAS₃₂₇₋₆₆₉) of CAS were purified with immobilized protein A (Pierce). Anti-CAS₃₂₇₋₆₆₉ was further purified by affinity chromatography by Ni-NTA chromatography as described for preparation of immunogen but used 1 M GuHCl without 2-mercaptoethanol (pH 4.5) as elution buffer. Fractions containing pure CAS protein were concentrated by Centricon 30 (Amicon) to 1.4 mg/ml, and 6 mg of the protein was then coupled to Affi-Gel 10 (Bio-Rad) activated with 0.1 M sodium acetate, pH 4.5/1 M GuHCl. Protein A purified anti-CAS₃₂₇₋₆₆₉ antibody from rabbit R46-I dialyzed against 0.1 M borate, pH 8.0/0.5 M NaCl was applied to the column equilibrated with dialysis buffer. After washing with dialysis buffer and 0.1 M sodium acetate, pH 4.8/0.5 M NaCl, the antibody was eluted in 1-ml steps with 0.2 M sodium-acetate (pH 2.5) and neutralized with 500 μ l of 1 M Tris-HCl (pH 9.0). BSA was added to a final concentration of 1 mg/ml. Antibody fractions (\approx 35 μ g of affinity-purified antibody per ml of immune serum) were dialyzed against PBS (pH 7.4) and stored at -20°C. The proteins were purified to near homogeneity (> 95% pure). About 6 mg of purified recombinant protein was obtained from a 1-liter culture of *E. coli* expressing the N-terminal CAS₁₋₂₈₄ fragment and 11 mg of the CAS₃₂₇₋₆₆₉ fragment per liter was obtained.

Western Blot Analysis, Immunofluorescence, and Immunohistochemical Staining. Western blot analysis was performed with the Vectastain ABC kit (Vector Laboratories) using biotinylated anti-rabbit IgG (H+L) as secondary antibody, avidin horseradish peroxidase, and 3,3'-diaminobenzidine (DAB) for color development. In all cases, only a single band was detected using anti-CAS antibodies produced against the CAS amino acids 1-284 when the serum was diluted either 1:1000 or 1:2000 in appropriate buffer. Control Western blots using the same amount of antigen and serum from pre-immunization rabbits at a 1:1000 dilution showed no detectable CAS band. Anti-CAS antibodies against CAS amino acids 327-669 showed a single band of detected protein on a Western blot when the serum was used at a 1:1000 dilution, and a weak band was detected when the serum was used at a 1:2000 dilution. The yield of affinity-purified anti-CAS₃₂₇₋₆₆₉ was \approx 1.5% of the total protein A-purified antibodies. Affinity purified anti-CAS₃₂₇₋₆₆₉ specially detects \leq 0.8 ng of CAS in Western blots developed with horseradish peroxidase and DAB as color substrate. A clear band was visible with 0.8 ng of purified recombinant CAS and 0.3 ng still showed a very weak band (data not shown). Control serum (at a dilution of 1:1000 or 1:2000) from the same rabbit before immunization showed no detectable signal on the Western blot.

When affinity purified anti-CAS antibodies against CAS amino acids 327-669 were used at a 1:2000 dilution on a Western blot, a strong single band of CAS protein was detected showing that the purification concentrated the antibodies relative to the serum which barely detected CAS when diluted 1:2000. The affinity purified anti-CAS antibodies were used for immunohistological analysis of CAS expression in cells and tumors and for fluorescence activated cell sorter (FACS) analyses presented below.

EXAMPLE 14: ANTI-CAS ANTIBODIES CAN DISTINGUISH RESTING (NORMAL) CELLS FROM PROLIFERATING AND TUMOR CELLS

Because we have shown that CAS mRNA is highly expressed in proliferating cells and tumor cell lines, it is feasible to assume, that also the CAS protein should be present at high levels in proliferating and tumor cells and at low levels in normal cells (with few exceptions, e.g. testis). Because of that, the detection of relative amounts of CAS protein can be of value for tumor diagnosis, e.g. to distinguish tumor tissues from normal tissues.

To demonstrate that the CAS protein level in cells is dependant on their proliferation status, we analyzed the CAS content in total cell extracts of resting human WI-38 fibroblasts (maintained in medium containing minimal serum) and of actively growing WI-38 cells (grown in medium containing 10-15% serum). Serum starvation leads to cell growth arrest. Actively growing WI-38 fibroblasts (in 10% serum containing medium) were shifted to 0.15% serum containing medium for 3 days. The cells were then returned to 10% serum containing medium, in which cells resumed proliferation until they reached confluency, when growth was again arrested. Protein samples were obtained from the cells under the different growth conditions and analyzed by immunoblot with polyclonal rabbit anti-CAS antibodies (see Example 13). The CAS protein was detected as a single protein band of about 100 kDa by Western blotting of total cellular protein using anti-CAS antibodies. FIG. 11 shows that CAS was expressed at a higher level in growing cells and at significantly lower levels in resting cells. Furthermore, the amount of CAS protein increased when the resting cells were induced to proliferate by addition of serum. Thus, detection of elevated CAS expression in cells is indicative of cell proliferation.

To analyze whether CAS protein levels are elevated in growing cancer cells relative to normal cells, we compared the CAS protein content in total cell extracts of human WI-38 fibroblasts (resting and actively growing, see above) with the CAS content in MCF-7 breast cancer cells, again using Western blots with anti-CAS antibodies. We found that CAS was expressed at a significantly higher level in the cancer cells compared to the "normal" cells, independent of whether the normal cells were growing or resting.

The high levels of CAS protein in cancer cells can also be detected using CAS-specific antibodies and secondary antibodies with fluorescent tags and immunofluorescence techniques well known to those skilled in the art. The presence of CAS protein in various cancer cells was detected by standard immunofluorescence and microscopic examination of MCF-7 breast cancer and A431 epidermoid cells. FACS analysis was used to detect CAS protein in CA46 lymphoma cells. When cells were stained with anti-CAS fluorescently labeled antibodies, the cells exhibited an average relative fluorescence about 100-fold greater than that of cells that were not stained with anti-CAS fluorescently labeled antibodies. In this FACS analysis, the relative fluorescence resulting from binding of the fluorescently-labeled anti-CAS antibody (on the Y axis) was graphed as a function of the DNA content of the cells determined by propidium iodide staining (on the X axis).

It will be appreciated by those skilled in the art that a kit for diagnosing cancer can be based on anti-CAS antibodies such as those described above. An antibody based diagnostic kit could use the anti-CAS antibodies in a variety of ways to detect cellular CAS protein in cancer cells. A kit could be used to detect binding of anti-CAS antibodies to CAS protein by directly measuring fluorescence from anti-CAS antibodies directly labeled with a fluorescent marking in a fluorescence immunoassay using techniques well known to those skilled in the art. Alternatively, a kit could

rely on other ways of detecting binding of anti-CAS antibody to CAS protein including detection of fluorescence using a fluorescently-labeled secondary antibody to bind to the anti-CAS antibody (based on specific binding to the anti-CAS antibody or cross-species antibody binding, dependent on either the variable or constant regions of the anti-CAS antibody, respectively). It will be appreciated by those skilled in the art that radiolabeling of the anti-CAS antibody or the secondary antibody that binds to the anti-CAS antibody could be effectively substituted for fluorescent labeling and the kit would rely on a radioimmunoassay (RIA). Furthermore, an enzyme substrate attached to the anti-CAS antibody or the secondary antibody that binds to the anti-CAS antibody could be used to detect binding by measuring the relative enzymatic activity of the bound antibodies in a enzyme linked immunosorbent assay (ELISA) using reactions well known to those skilled in the art. Those skilled in the art will further appreciate that an antibody-based kit could use latex beads, either directly linked to the anti-CAS antibody or linked to the secondary antibody that binds to the anti-CAS antibody. Anti-CAS antibodies could further be used in a kit in which the anti-CAS antibodies serve as part of a sandwich immunoassay in which the CAS protein is immobilized to a solid support for detection. It will be understood that these examples are nonlimiting and additional methods of antibody-based detection of CAS protein are also contemplated for use in a kit of the present invention.

We conclude that elevated CAS expression in cells is indicative for cell proliferation, and that higher levels of CAS protein are detectable in cancer cells, compared to most normal cells.

EXAMPLE 15: LOCALIZATION OF CAS PROTEIN IN CELLULAR MICROTUBULES AND METAPHASE SPINDLES

Using immuno-assays, the CAS protein was localized to the cytoplasmic fraction of cells, primarily in microtubules and in mitotic spindles of metaphase cells. Western blot assays were done essentially as described in Example 13. For immunofluorescence, affinity-purified anti-CAS preparation (10 μ g/ml) or antitubulin monoclonal antibody (10 μ g/ml) (clone YL 1/2-supernatant; Accurate Scientific, Westbury, NY) were incubated with MCF-7, A431, OVCAR3, or KB cells grown for 1 day in 35-mm dishes to subconfluency. Cells were fixed and permeabilized using 3.7% formaldehyde in PBS for 10 min followed by inclusion of 0.1% saponin in all subsequent incubations. Bound antibody was detected with goat anti-rat IgG rhodamine for anti-tubulin antibody and with goat anti-rabbit-rhodamine for anti-CAS antibody. For double-label immunofluorescence, MCF-7 cells were fixed and processed as described for single-label immunofluorescence. Cells were then sequentially incubated with rabbit anti-CAS and rat monoclonal anti-tubulin, followed by affinity-purified goat anti-rabbit rhodamine having minimal cross-reaction to rat, and goat anti-rat fluorescein having minimal cross-reaction to rabbit (Jackson ImmunoResearch). Controls demonstrated that the two antibody systems showed no cross-reactivity and no cross-detection between fluorescence channels. Frozen tissue samples from the Hollings Cancer Center Tumor Bank Collection at the Medical University of South Carolina were cryostat sectioned and processed for immunohistochemical staining.

Cell Fractionation and Preparation of Cytoskeleton. MCF-7 or A431 cells (2×10^6) were chilled to 4°C, washed three times with ice-cold Dulbecco's PBS without Ca^{2+} / Mg^{2+} , scraped from the flasks, and suspended in 1 ml of hypotonic buffer (25 mM Tris-HCl, pH 7.4/2 mM MgCl_2 /1 mM EDTA) for 15 min on ice. Then the suspension was homogenized (20 strokes in a Dounce homogenizer) and centrifuged (1,000 $\times g$ at 4°C for 5 min). The pellet is the nuclear fraction. The supernatant was centrifuged (100,000 $\times g$ at 4°C for 60 min.) and the supernatant from this

centrifugation step is the cytosolic fraction. The pellet from the second centrifugation step, which contains mainly membranes, was resuspended in hypotonic buffer. Cytoskeletons of MCF-7 cells grown in 35-mm poly(L-lysine) dishes were prepared by washing cells attached to the dishes three times with 0.1 M Mes/1 mM EGTA/1 mM MgCl₂/4% PEG 8000, pH 6.5, followed by incubation for 20 min with 0.2% Triton X-100 in the same buffer, and washing three times with PBS. The cytoskeletons still attached to the dishes were fixed with 3.7% formaldehyde for 10 min, washed three times with PBS, and subjected to immunofluorescence.

CAS is an abundant cytosolic protein. CAS cDNA contains an open reading frame encoding a protein of about 100-kDa. To analyze the specificity of the antibody preparation and to confirm the open reading frame deduced from CAS cDNA, we analyzed total cell extracts of MCF-7 and A431 cells by Western blots. We detected an approximately 100-kDa band in the extracts with immune serum but not with preimmune serum. Using immunoblot analyses of total cell protein of MCF-7 cells on 4-15% SDS gels with anti-CAS₁₋₂₈₄ and anti-CAS₃₂₇₋₆₆₉ antibodies, we found that both anti-CAS antisera specifically detected an approximately 100-kDa protein, as predicted from the cDNA sequence, while the corresponding preimmune sera did not.

To evaluate the cellular localization of CAS protein, we homogenized MCF-7 and A431 cells and prepared fractions containing membranes, cytosol, and nuclei. Western blot analysis with anti-CAS₁₋₂₈₄ and anti-CAS₃₂₇₋₆₆₉ antibodies showed that CAS is present exclusively in the cytosolic fraction.

To obtain an estimate of the number of CAS molecules per cell, we compared the CAS signal intensity in the cytosolic fraction of MCF-7 cells with the signal intensity of known amounts of recombinant CAS. We found that the signal from protein present in 10⁴ cells corresponded to about 1 ng of CAS. Thus, one MCF-7 cell contains about 0.1 pg of CAS (i.e., $\approx 10^{-18}$ mol or $\approx 600,000$ molecules), indicating that CAS is an abundant protein. Taking into account the variations between different immunoblot analyses and extract preparations, we estimate the number of CAS molecules is between 2×10^5 and 1×10^6 per cell in actively growing cells.

CAS is associated with microtubules. To obtain more information about the intracellular distribution of CAS, we analyzed permeabilized MCF-7, A431, OVCAR3, and KB cells by immunofluorescence with affinity-purified anti-CAS₃₂₇₋₆₆₉ antibody. Anti-CAS antibody shows a diffuse background staining and clear staining of structures that form an intracellular network in all cell lines. This distribution resembles that of microtubules. We therefore compared cells stained with anti-CAS antibody to cells stained with anti-tubulin antibody and found that CAS is distributed in cells in a pattern similar to tubulin, indicating that CAS is associated with or part of microtubules. Anti-CAS staining of metaphase MCF-7 cells showed that CAS is also associated with the chromosome segregation spindle in mitotic cells. The spindle and the intercellular bridge between cells that have just divided stained strongly. The intracellular distribution of CAS, as defined with anti-CAS₃₂₇₋₆₆₉ antibody, was confirmed with protein A-purified anti-CAS₁₋₂₈₄ antibody, which showed the same intracellular CAS distribution and localization. Also, a separate double-label immunofluorescence experiment was performed which demonstrated that the tubular structures detected by anti-CAS and anti-tubulin antibodies are the same.

CAS is not an integral part of the microtubules and is not tightly bound to microtubules. Possible explanations for a tubulin-like intracellular distribution of CAS, as observed by immunofluorescence with anti-CAS

antibody, are that (1) the polyclonal antibody preparations crossreact with tubulin, (2) CAS is an integral part of microtubules, or (3) CAS is associated with but not part of microtubules. To rule out the possibility that our antibody preparations cross-react with tubulin, we analyzed by Western blot analysis a tubulin preparation (T-4925; Sigma) derived from bovine brain, which contains about 15% microtubule-associated proteins (MAP). The gel was loaded with 60 μ g of tubulin, and no reactivity of anti-CAS with tubulin was detected. Also, anti-CAS₂₂₇₋₆₆₉ antibody did not react with tubulin-associated proteins in immunoblots. Although our antibodies were prepared against human CAS, we would expect to detect bovine CAS, because the cDNA sequence is highly conserved between humans and yeast, and our antibody detected mouse CAS. Thus, our antibody preparations do not cross-react with tubulin or with MAP that are present in this tubulin preparation. Furthermore, sera from animals that were independently immunized with different parts of CAS showed the same distribution pattern and the same protein size, making it unlikely that the signals observed were due to cross-reactivity. Thus, CAS is either an integral part of microtubules or is associated with microtubules.

To distinguish between these possibilities, we chose two approaches. First, we isolated cytoskeletal microtubules by an EGTA/Mg²⁺/Triton X-100 method, which retains the microtubule network but removes loosely associated proteins. If CAS is an integral part of microtubules or very tightly bound to microtubules, the cytoskeletal preparation should stain with anti-CAS and with anti-tubulin antibodies. If CAS is associated loosely to microtubules, the anti-CAS staining pattern should be weaker than with anti-tubulin. Our immunofluorescent results showed cytoskeletal preparations of MCF-7 cells stained with anti-CAS₂₂₇₋₆₆₉ and anti-tubulin antibodies but the distribution of CAS and tubulin was different. That is, anti-tubulin strongly stained the microtubule structures but anti-CAS produced a weak diffuse image. Only highly stable structures such as intercellular bridges still showed some anti-CAS staining. These results indicate that although CAS is associated with the microtubular cytoskeleton, as seen by whole cell staining, it is not an integral part of the cytoskeleton because most of the CAS protein was removed from the cytoskeleton during the EGTA/Triton X-100 preparation.

We also analyzed CAS distribution in MCF-7 cells that were treated with vincristine or taxol. Vincristine treatment results in formation of tubulin paracrystals and taxol produces tubulin bundles. If CAS were an integral part of microtubules, we would expect similar images of vincristine- and taxol-treated cells stained with anti-tubulin or anti-CAS. However, we found that anti-CAS produced a strong diffuse cytoplasmic staining and only a weak immunofluorescence signal associated with paracrystals and bundles, in contrast to strong anti-tubulin staining of paracrystals and microtubule bundles in vincristine- and taxol-treated cells. Thus, CAS is associated with but not an integral part of microtubules and CAS that is bound to microtubules is released by the EGTA/Triton X-100 incubation during cytoskeleton preparation and in vincristine- and taxol-treated cells.

EXAMPLE 16: LOCALIZATION OF CAS PROTEIN IN LYMPHOID NEOPLASMS

Because CAS expression is associated with cell proliferation and apoptosis, factors known to play a role in lymphoma development, and CAS is highly expressed in some leukemia and lymphoma cell lines (e.g., HL-60, K-562, MOLT-4 and Raji), the role of CAS expression in lymphoma development was investigated. CAS protein distribution in normal tonsils and different lymphoma subtypes was analyzed by immunohistochemistry using antibodies against CAS (as described in Examples 13-15).

Formalin or B5 fixed and paraffin embedded biopsies from 26 cases of malignant lymphomas were selected from the histopathology files of the Hematopathology Section, Laboratory of Pathology, National Cancer Institute, National Institutes of Health (Bethesda, MD, USA). The lymphomas were classified according to the Revised European-American Classification of Lymphoid Neoplasms (REAL; Harris, N.L. et al., *Blood* 1:1361-1392, 1994). The cases included five follicular lymphomas (FL), four B-cell chronic lymphoid leukemias (B-CLL), six diffuse large B-cell lymphomas (DLBCL), two peripheral and three precursor (lymphoblastic) T-cell lymphomas (TCL), four anaplastic large cell lymphomas of T- and null-cell phenotypes (ALCL), and two cases of Hodgkin's disease (HD). All cases had been immunophenotyped either in paraffin or frozen sections.

Polyclonal antibodies against CAS were raised in rabbits immunized with recombinant CAS protein and purified essentially as described in Example 13.

Five μ m paraffin sections were mounted on Fisherbrand/Plus Superfrost slides (Fisher Scientific, Pittsburg, PA). After blocking endogenous peroxidase activity, a wet heat antigen retrieval was performed by boiling in a microwave pressure cooker (Nordic Ware, Minneapolis, MN) in 0.01 M citrate buffer, pH 6.0, at 800 W for 40 min. The sections were rinsed in 0.05 M Tris-HCL saline, pH 7.6 (TBS) containing 5% fetal calf serum (FCS) for 15 min. Then sections were incubated overnight with anti-CAS antibody (0.1 μ g/ml in TBS containing 20% FCS and 0.1% NaN₃) at room temperature (RT). Biotinylated anti-rabbit anti-mouse secondary antibody was used for detection of anti-CAS binding, and the peroxidase reaction was developed using well known techniques (e.g., as utilized in the StreptABComplex/HRP Duet Kit and DAKO AEC Substrate System, DAKO Corporation, Carpinteria, CA). For simultaneous staining of CAS and Ki-67 proteins, biotinylated goat anti-rabbit immunoglobulins secondary antibody (1/400 dilution) and streptavidin-peroxidase conjugate (1/600 dilution) were used for the CAS immunoreaction (both reagents from DAKO). After the development of the peroxidase reaction, the sections were incubated for 60 min at RT with a mouse anti-Ki-67 monoclonal antibody (M1B1, at 1/10 dilution; Immunotech, Westbrook, ME) and then goat anti-mouse immunoglobulin (1/25 dilution) and a mouse APAAP complex (1/50 dilution; both reagents from DAKO) were applied. The second immunoreaction was visualized with BCIP/NBT (Boehringer Mannheim). All sections were counterstained with hematoxylin and mounted with Glycergel™ (DAKO). Immunohistochemical staining with anti-CAS antibody of fixed and paraffin-embedded specimens compared to staining of frozen tissue slides revealed similar staining patterns for both specimen types. Therefore, all further experiments were done on formalin-fixed, paraffin embedded tissue.

Normal tonsil stained with anti-CAS antibody and double stained with anti-CAS/Ki-67 antibodies showed a polarized CAS staining of about 20% of the cells, condensed to the dark zone of the follicle. The strongest staining was found in the large cells. In contrast, the majority of mantle zone and interfollicular cells did not stain for CAS, except for some single larger cells having moderate to strong staining. The staining was confined to the cytoplasm of the positive cells. Anti-CAS/anti-Ki-67 double staining demonstrated that most of the strongly CAS-positive cells were also positive for Ki-67 but about 10% to 15% of the strongly CAS-positive cells did not stain for Ki-67. Furthermore, almost all weakly CAS-positive cells were negative for Ki-67.

Expression and distribution of CAS was analyzed in 26 lymphomas (see Table 3). Staining of CAS protein in follicular lymphomas demonstrated a very intense cytoplasmatic reaction in 50% to 60% of the atypical larger cells.

Compared to the distribution of CAS-positive cells in normal tonsils, these strongly CAS-positive lymphoma cells were stained in a more scattered and irregular pattern throughout the tumor, although most of the CAS-positive cells were confined to the follicles but not in a polarized manner. Anti-CAS/anti-Ki-67 double staining showed a good correlation between CAS and Ki-67 expression but about 10% more CAS-positive cells were detected compared to Ki-67 positive cells. The small cells of the follicles were CAS-negative or showed weaker staining.

In histologically aggressive lymphomas (e.g., ALCL and DLBCL), we found that 70% of the multinucleated large tumor cells were CAS-positive. Most of these cells were also positive for Ki-67, but about 10% of the CAS-positive cells were negative for Ki-67. In addition to ALCL and DLBCL, we analyzed B-CLL, TCL and Hodgkin's disease lymphomas.

The results of immunostaining of the lymphoma specimens are summarized in Table 3. B-CLL showed a generally weaker staining with anti-CAS, staining about 5% to 10% of the malignant cells; anti-CAS/anti-Ki-67 doublestaining showed the same results. These weakly CAS-positive cells were scattered throughout the atypical infiltrate and are most likely paraimmunoblasts. In TCL, about 15% to 20% of the malignant cells were weakly CAS-positive, which reflected the staining pattern of Ki-67. The Hodgkin's disease cases revealed a strong staining for CAS and Ki-67 in 80% of the Reed-Sternberg and mononuclear variant cells.

The aggressive NHL and HD lymphomas displayed the strongest CAS-positive staining, with up to 80% of the malignant cells staining. The CAS and Ki-67 expression patterns are almost congruent within the different lymphoma subtypes, but a subpopulation of some malignant cells that were CAS-positive but Ki-67-negative was observed.

These results show that CAS protein is abundant in benign proliferating lymphocytes of the tonsillar follicles. CAS was predominantly in the larger malignant cells of the studied lymphoid tumors and thus may be used as a marker for diagnosis and prognosis testing of these types of lymphomas using similar antibody staining techniques as described above. The reagents for detecting CAS in suspected lymphoma tissue by immunofluorescent methods may be supplied in a kit for diagnostic use.

Table 3: CAS Staining in Normal Tonsils and Various Lymphomas

	Tissue	Number	% CAS-staining Cells	Tissue Distribution
	Tonsil	5	20	polarized in dark zone of follicle
	Follicular Lymphoma	5	50-60	scattered through neoplastic nodules
5	Diffuse Large B-cell & Anaplastic Large Cell Lymphomas	10	60-70	in multinucleated large cells
	B-cell Chronic Lymphoid Leukemia	4	5-10	weak & scattered through infiltrate
10	T-cell Lymphoma	5	15-20	weak & scattered through infiltrate
	Hodgkin's Disease	2	80	Reed-Sternberg & mononuclear variants

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: The United States, As Represented by the Secretary,
Department of Health and Human Services

(ii) TITLE OF THE INVENTION: CELLULAR APOPTOSIS SUSCEPTIBILITY
PROTEIN (CSP) AND ANTISENSE CSP

(iii) NUMBER OF SEQUENCES: 14

(iv) CORRESPONDENCE ADDRESS:

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ Version 1.5

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3180 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTGCGCCCAT TTTGCCGGGG TTTGAATGTG AGGCGGAGCG GCGGCAGGAG CGGATAGTGC
CAGCTACGGT CCGCGGCTGG GGTTCCTCC TCCGTTTCTG TATCCCCACG AGATCCTATA

60
120

GCAATGGAAC	TCAGCGATGC	AAATCTGCAA	ACACTAACAG	AATATTTAAA	GAAAACACTT	180
GATCCTGATC	CTGCCATCCG	ACGTCCAGCT	GAGAAATTTT	TTGAATCTGT	TGAAGGAAAT	240
CAGAATTATC	CACTGTTGCT	TTTGACATTA	CTGGAGAAGT	CCCAGGATAA	TGTTATCAAA	300
GTATGTGCTT	CAGTAACATT	CAAAAACAT	ATTTAAAGGA	ACTGGAGAAT	TGTTGAAGAT	360
GAACCAAACA	AAATTTGTGA	AGCCGATCGA	GTGGCCATTA	AAGCCAACAT	AGTGCACCTG	420
ATGCTTAGCA	GCCCAGAGCA	AATTCAGAAG	CAGTTAAGTG	ATGCAATTAG	CATTATTGGC	480
AGAGAAGATT	TTCCACAGAA	ATGGCCTGAC	TTGCTGACAG	AAATGGTGAA	TCGCTTTCAG	540
AGTGGAGATT	TCCATGTTAT	TAATGGAGTC	CTCCGTACAG	CACATTCATT	ATTTAAAAGA	600
TACCGTCATG	AATTTAAGTC	AAACGAGTTA	TGGACTGAAA	TTAAGCTTGT	TCTGGATGCC	660
TTTGCTTTGC	CTTTGACTAA	TCTTTTAAAG	GCCACTATTG	AACTCTGCAG	TACCCATGCA	720
AATGATGCCT	CTGCCCTGAG	GATTCTGTTT	TCTTCCCTGA	TCCTGATCTC	AAAATTGTTC	780
TATAGTTTAA	ACTTTTCAGGA	TCTCCCTGAA	TTTTGGGAAG	GTAATATGGA	AACTTGGATG	840
AATAATTTCC	ATACTCTCTT	AACATTGGAT	AATAAGCTTT	TACAAACTGA	TGATGAAGAG	900
GAAGCCGGCT	TATTGGAGCT	CTTAAAATCC	CAGATTTGTG	ATAATGCCGC	ACTCTATGCA	960
CAAAAGTACG	ATGAAGAATT	CCAGCGATAC	CTGCCTCGTT	TTGTTACAGC	CATCTGGAAT	1020
TTACTAGTTA	CAACGGGTCA	AGAGGTTAAA	TATGATTTGT	TGGTAAGTAA	TGCAATTCAA	1080
TTTCTGGCTT	CAGTTTGTGA	GAGACCTCAT	TATAAGAATC	TATTTGAGGA	CCAGAACACG	1140
CTGACAAGTA	TCTGTGAAAA	GGTTATTGTG	CCTAACATGG	AATTTAGAGC	TGCTGATGAA	1200
GAAGCATTGG	AAGATAAATC	TGAGGAGTAC	ATAAGGAGAG	ATTTGAGAGG	ATCTGATATT	1260
GATACTAGAC	GCAGGGCTGC	TTGTGATCTG	GTACGAGGAT	TATGCAAGTT	TTTTGAGGGA	1320
CCTGTGACAG	GAATCTTCTC	TGGTTATGTT	AATTCCATGC	TGCAGGAATA	CGCAAAAAAT	1380
CCATCTGTCA	ACTGGAACA	CAAAGATGCA	GCCATCTACC	TAGTGACATC	TTTGGCATCA	1440
AAAGCCCAAA	CACAGAAGCA	TGGAATTACA	CAAGCAAATG	AACCTGTAAA	CCTAACTGAG	1500
TTCTTTGTGA	ATCACATCCT	CCCTGATTTA	AAATCAGCTA	ATGTGAATGA	ATTTCTGTGC	1560
CTTAAAGCTG	ACGGTATCAA	ATATATTATG	ATTTTGTAGAA	ATCAAGTGCC	AAAAGAACAT	1620
CTTTTAGTCT	CGATTCTCT	CTTGATTAA	CATCTTCAAG	CTGGAAGTAT	TGTTGTTTCAT	1680
ACTTACGCAG	CTCATGCTCT	TGAACGGCTC	TTTACTATGC	GAGGGCCTAA	CAATGCCACT	1740
CTCTTTACAG	CTGCAGAAAT	CGCACCGTTT	GTTGAGATT	TGCTAACAAA	CCTTTTCAAA	1800
GCTCTACAC	TTCCTGGCTC	TTTCAAAAA	GAATATATTA	TGAAAGCTAT	CATGAGAAGT	1860
TTTTCTCTCC	TACAAGAAGC	CATAATCCCC	TACATCCCTA	CTCTCATCAC	TCAGCTTACA	1920
CAGAAGCTAT	TAGCTGTTAG	TAAGAACCCA	AGCAAACCTC	ACTTTAATCA	CTACATGTTT	1980
GAAGCAATAT	GTTTATCCAT	AAGAATAACT	TGCAAAGCTA	ACCCTGCTGC	TGTTGTAAAT	2040
TTTGAGGAGG	CTTTGTTTTT	GGTGTTTACT	GAAATCTTAC	AAAATGATGT	GCAAGAATTT	2100
ATTCCATACG	TCTTTCAAGT	GATGTCTTTG	CTTCTGGAAG	CACACAAAAA	TGACATCCCG	2160
TCTTCTTATA	TGGCCTTATT	TCCTCATCTC	CTTCAGCCAG	TGCTTTGGGA	AAGAACAGGA	2220
AATATTCTTG	CTCTAGTGAG	GCTTCTTCAA	GCATTCTTAG	AACGCGGTTT	AAACACAATA	2280
GCAAGTGCTG	CAGCTGACAA	AATTCTCTGG	TTACTAGGTG	TCTTTCAGAA	GCTGATTGCA	2340
TCCAAAGCAA	ATGACCACCA	AGGTTTTTAT	CTTCTAAACA	GTATAATAGA	GCACATGCCT	2400
CCTGAATCAG	TTGACCAATA	TAGGAAACAA	ATCTTCATTC	TGCTATTCCA	GAGACTTCAG	2460
AATTCCAAAA	CAACCAAGTT	TATCAAGAGT	TTTTTAGTCT	TTATTAATTT	GTATTGCATA	2520
AAATATGGGG	CAC TAGCACT	ACAAGAAATA	TTTGATGGTA	TACAACCAAA	AATGTTTGGA	2580
ATGGTTTTGG	AAAAAATTAT	TATTCCTGAA	ATTCAGAAGG	TATCTGGAAG	TGTAGAGAAA	2640
AAGATCTGTG	CGGTTGGCAT	AACCAACTTA	CTAACAGAAT	GTCCCCCAAT	GATGGACACT	2700
GAGTATACCA	AACGTGTGAC	TCCATTATTA	CAGTCTTTGA	TTGGTCTTTT	TGAGTTACCC	2760
GAAGATGATA	CCATTCTCTG	TGAGGAACAT	TTTATTGACA	TAGAAGATAC	ACCAGGATAT	2820
CAGACTGCCT	TCTCACAGTT	GGCATTGTCT	GGGAAAAAAG	AGCATGATCC	TGTAGGTCAA	2880
ATGGTGAATA	ACCCCAAAAT	TCACCTGGCA	CAGTCACTTC	ACATGTTGTC	TACCGCCTGT	2940
CCAGGAAGGG	TTCCATCAAT	GGTGAGCACC	AGCCTGAATG	CAGAAGCGCT	CCAGTATCTC	3000
CAAGGGTACC	TTCAGGCAGC	CAGTGTGACA	CTGCTTTAAA	CTGCATTTTT	CTAATGGGCT	3060
AAACCCAGAT	GGTTTCCTAG	GAAATCACAG	GCTTCTGAGC	ACAGCTGCAT	TAAAACAAAG	3120
GAAGTTTTCC	TTTTGAACIT	GTCACGAAAA	AAAAAATAAA	AAAAAATAAA	AAAAAATAAA	3180

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 971 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Glu Leu Ser Asp Ala Asn Leu Gln Thr Leu Thr Glu Tyr Leu Lys
 1 5 10 15
 Lys Thr Leu Asp Pro Asp Pro Ala Ile Arg Arg Pro Ala Glu Lys Phe
 20 25 30
 Leu Glu Ser Val Glu Gly Asn Gln Asn Tyr Pro Leu Leu Leu Leu Thr
 35 40 45
 Leu Leu Glu Lys Ser Gln Asp Asn Val Ile Lys Val Cys Ala Ser Val
 50 55 60
 Thr Phe Lys Asn Tyr Ile Lys Arg Asn Trp Arg Ile Val Glu Asp Glu
 65 70 75 80
 Pro Asn Lys Ile Cys Glu Ala Asp Arg Val Ala Ile Lys Ala Asn Ile
 85 90 95
 Val His Leu Met Leu Ser Ser Pro Glu Gln Ile Gln Lys Gln Leu Ser
 100 105 110
 Asp Ala Ile Ser Ile Ile Gly Arg Glu Asp Phe Pro Gln Lys Trp Pro
 115 120 125
 Asp Leu Leu Thr Glu Met Val Asn Arg Phe Gln Ser Gly Asp Phe His
 130 135 140
 Val Ile Asn Gly Val Leu Arg Thr Ala His Ser Leu Phe Lys Arg Tyr
 145 150 155 160
 Arg His Glu Phe Lys Ser Asn Glu Leu Trp Thr Glu Ile Lys Leu Val
 165 170 175
 Leu Asp Ala Phe Ala Leu Pro Leu Thr Asn Leu Phe Lys Ala Thr Ile
 180 185 190
 Glu Leu Cys Ser Thr His Ala Asn Asp Ala Ser Ala Leu Arg Ile Leu
 195 200 205
 Phe Ser Ser Leu Ile Leu Ile Ser Lys Leu Phe Tyr Ser Leu Asn Phe
 210 215 220
 Gln Asp Leu Pro Glu Phe Trp Glu Gly Asn Met Glu Thr Trp Met Asn
 225 230 235 240
 Asn Phe His Thr Leu Leu Thr Leu Asp Asn Lys Leu Leu Gln Thr Asp
 245 250 255
 Asp Glu Glu Glu Ala Gly Leu Leu Glu Leu Leu Lys Ser Gln Ile Cys
 260 265 270
 Asp Asn Ala Ala Leu Tyr Ala Gln Lys Tyr Asp Glu Glu Phe Gln Arg
 275 280 285
 Tyr Leu Pro Arg Phe Val Thr Ala Ile Trp Asn Leu Leu Val Thr Thr
 290 295 300
 Gly Gln Glu Val Lys Tyr Asp Leu Leu Val Ser Asn Ala Ile Gln Phe
 305 310 315 320
 Leu Ala Ser Val Cys Glu Arg Pro His Tyr Lys Asn Leu Phe Glu Asp
 325 330 335
 Gln Asn Thr Leu Thr Ser Ile Cys Glu Lys Val Ile Val Pro Asn Met
 340 345 350
 Glu Phe Arg Ala Ala Asp Glu Glu Ala Phe Glu Asp Asn Ser Glu Glu
 355 360 365
 Tyr Ile Arg Arg Asp Leu Glu Gly Ser Asp Ile Asp Thr Arg Arg Arg
 370 375 380
 Ala Ala Cys Asp Leu Val Arg Gly Leu Cys Lys Phe Phe Glu Gly Pro
 385 390 395 400
 Val Thr Gly Ile Phe Ser Gly Tyr Val Asn Ser Met Leu Gln Glu Tyr
 405 410 415
 Ala Lys Asn Pro Ser Val Asn Trp Lys His Lys Asp Ala Ala Ile Tyr
 420 425 430
 Leu Val Thr Ser Leu Ala Ser Lys Ala Gln Thr Gln Lys His Gly Ile
 435 440 445
 Thr Gln Ala Asn Glu Leu Val Asn Leu Thr Glu Phe Phe Val Asn His
 450 455 460
 Ile Leu Pro Asp Leu Lys Ser Ala Asn Val Asn Glu Phe Pro Val Leu
 465 470 475 480
 Lys Ala Asp Gly Ile Lys Tyr Ile Met Ile Phe Arg Asn Gln Val Pro
 485 490 495
 Lys Glu His Leu Leu Val Ser Ile Pro Leu Leu Ile Asn His Leu Gln

Ala	Gly	Ser	500	Val	Val	His	Thr	505	Tyr	Ala	Ala	His	Ala	510	Leu	Glu	Arg
Leu	Phe	Thr	515	Met	Arg	Gly	Pro	520	Asn	Asn	Ala	Thr	Leu	525	Phe	Thr	Ala
530	Glu	Ile	Ala	Pro	Phe	Val	Glu	535	Ile	Leu	Leu	Thr	540	Asn	Leu	Phe	Lys
545	Leu	Thr	Leu	Pro	Gly	Ser	Ser	550	Glu	Asn	Glu	Tyr	555	Ile	Met	Lys	Ala
560	Met	Arg	Ser	565	Phe	Ser	Leu	Leu	Gln	Glu	Ala	Ile	570	Ile	Pro	Tyr	Ile
575	Thr	Leu	Ile	580	Thr	Gln	Leu	Thr	Gln	Lys	Leu	Leu	585	Ala	Val	Ser	Lys
590	Pro	Ser	Lys	595	Pro	His	Phe	Asn	His	Tyr	Met	Phe	600	Glu	Ala	Ile	Cys
605	610	Ser	Ile	Arg	Ile	Thr	Cys	Lys	Ala	Asn	Pro	Ala	615	Ala	Val	Val	Asn
620	625	Glu	Glu	Ala	Leu	Phe	Leu	Val	Phe	Thr	Glu	Ile	630	Leu	Gln	Asn	Asp
635	Gln	Glu	Phe	Ile	Pro	Tyr	Val	Phe	Gln	Val	Met	Ser	635	Leu	Leu	Glu	640
645	650	Thr	His	Lys	Asn	Asp	Ile	Pro	Ser	Ser	Tyr	Met	650	Ala	Leu	Phe	Pro
655	660	Leu	Leu	Gln	Pro	Val	Leu	Trp	Glu	Arg	Thr	Gly	665	Asn	Ile	Pro	Ala
670	675	Val	Arg	Leu	Leu	Gln	Ala	Phe	Leu	Glu	Arg	Gly	680	Ser	Asn	Thr	Ile
685	690	Ser	Ala	Ala	Ala	Asp	Lys	Ile	Pro	Gly	Leu	Leu	695	Gly	Val	Phe	Gln
700	705	Leu	Ile	Ala	Ser	Lys	Ala	Asn	Asp	His	Gln	Gly	705	Phe	Tyr	Leu	Leu
710	715	Ser	Ile	Ile	Glu	His	Met	Pro	Pro	Glu	Ser	Val	715	Asp	Gln	Tyr	Arg
720	725	Gln	Ile	Phe	Ile	Leu	Leu	Phe	Gln	Arg	Leu	Gln	725	Ser	Lys	Thr	Thr
730	735	Lys	Phe	Ile	Lys	Ser	Phe	Leu	Val	Phe	Ile	Asn	735	Leu	Tyr	Cys	Ile
740	745	Tyr	Gly	Ala	Leu	Ala	Leu	Gln	Glu	Ile	Phe	Asp	745	Gly	Ile	Gln	Pro
750	755	Met	Phe	Gly	Met	Val	Leu	Glu	Lys	Ile	Ile	Pro	755	Glu	Ile	Gln	Lys
760	765	Val	Ser	Gly	Asn	Val	Glu	Lys	Lys	Ile	Cys	Ala	765	Val	Gly	Ile	Thr
770	775	Leu	Leu	Thr	Glu	Cys	Pro	Pro	Met	Met	Asp	Thr	775	Glu	Tyr	Thr	Lys
780	785	Trp	Thr	Pro	Leu	Leu	Gln	Ser	Leu	Ile	Gly	Leu	780	Phe	Glu	Leu	Pro
785	790	Asp	Asp	Thr	Ile	Pro	Asp	Glu	Glu	His	Phe	Ile	785	Asp	Ile	Glu	Asp
795	800	Pro	Gly	Tyr	Gln	Thr	Ala	Phe	Ser	Gln	Leu	Ala	795	Phe	Ala	Gly	Lys
805	810	Glu	His	Asp	Pro	Val	Gly	Gln	Met	Val	Asn	Asn	805	Pro	Lys	Ile	His
815	820	Ala	Gln	Ser	Leu	His	Met	Leu	Ser	Thr	Ala	Cys	815	Pro	Gly	Arg	Val
825	830	Ser	Met	Val	Ser	Thr	Ser	Leu	Asn	Ala	Glu	Ala	825	Leu	Gln	Tyr	Leu
835	840	Gly	Tyr	Leu	Gln	Ala	Ala	Ser	Val	Thr	Leu	Leu	835	Gly	Leu	Gln	960
845	850	Thr	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	845	Leu	Leu	Leu	970
855	860	Thr	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	855	Leu	Leu	Leu	970
865	870	Thr	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	865	Leu	Leu	Leu	970
875	880	Thr	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	875	Leu	Leu	Leu	970
885	890	Thr	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	885	Leu	Leu	Leu	970
895	900	Thr	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	895	Leu	Leu	Leu	970
905	910	Thr	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	905	Leu	Leu	Leu	970
915	920	Thr	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	915	Leu	Leu	Leu	970
925	930	Thr	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	925	Leu	Leu	Leu	970
935	940	Thr	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	935	Leu	Leu	Leu	970
945	950	Thr	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	945	Leu	Leu	Leu	970
955	960	Thr	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	955	Leu	Leu	Leu	970
965	970	Thr	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	965	Leu	Leu	Leu	970

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
 (B) TYPE: nucleic acid

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTGGTTCGGC CCACCTCTGA AGGTTCCAGA ATCGATAG

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- (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TAATGAGGTC TCTCACAAA

19

- (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GACATCCCGT CTTCTATAT G

21

- (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AAGAAGCCTC ACTAGAGCAG GA

22

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TGTGAAGCCG ATCGAGTGGC

20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TTCAGGGACA TCCTGAAAGT

20

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCCCACTGAT GGACACTGA

19

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CTCACCATTG ATGGAACCC

19

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GAGATCCTAT ACATATGGAA CTCCAGCGAT G
31

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TATCGCTGGA ATTCTCATCC TAC

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(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CAGTTTGTCATATGCCTCAT TATAAAATC

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(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GAAGCAAAGA ATTCACTTGA AAGACGTATG

WHAT IS CLAIMED IS:

1. An isolated cDNA encoding a human CAS protein having the sequence of SEQ ID NO:2.
2. An isolated cDNA encoding a portion of a human CAS protein consisting of amino acids at positions 700-845 of SEQ ID NO:2.
- 5 3. The isolated cDNA of Claim 1, wherein said cDNA has the sequence of SEQ ID NO:1.
4. The isolated cDNA of Claim 2, wherein said cDNA consists of nucleotides 2100 to 2536 of SEQ ID NO:1.
5. The isolated cDNA of Claim 2, wherein said cDNA sequence is a single-stranded antisense CAS gene sequence.
- 10 6. A purified and isolated human CAS protein having the amino acid sequence of SEQ ID NO:2.
7. A purified and isolated human CAS protein consisting of the amino acid sequence corresponding to amino acids at positions 700-845 of SEQ ID NO:2.
8. A method of detecting cancerous cells in a biological sample which comprises cells from a human subject, said method comprising:
 - 15 obtaining said biological sample from said subject;
measuring expression of a human CAS gene in said sample; and comparing the measured amount of expression with the amount of expression of said human CAS gene in a normal, noncancerous sample of the same type, wherein measurement at a level higher than expression of said CAS gene in said normal, noncancerous sample indicates the presence of cancerous cells.
- 20 9. The method of Claim 8, wherein CAS gene expression is measured by detecting the level of CAS mRNA in said cells.
10. The method of Claim 9, wherein said CAS mRNA is detected by hybridization with a complementary DNA or by a polymerase chain reaction.
11. The method of Claim 8, wherein CAS gene expression is measured by detecting the level of CAS
25 protein in cells.
12. The method of Claim 8, wherein said CAS protein is detected by binding of antibody that specifically recognizes CAS protein.
13. A method of detecting cancerous cells in a biological sample which comprises cells from a human subject, said method comprising:
 - 30 obtaining said biological sample from said subject;
measuring the number of copies of a human CAS gene present in said cells; and
comparing the measured number of copies with the number of copies of said human CAS gene in a normal, noncancerous cell of the same type, wherein measurement at a level higher than the number of copies of said CAS gene in said normal, noncancerous sample indicates the presence of cancerous cells.

14. The method of Claim 13, wherein the number of copies of a CAS gene is measured by detecting the amount of hybridization of a single stranded polynucleotide having a sequence complementary to or the same as SEQ ID NO: 1 or any portion of 25 or more consecutive nucleotides therein.

15. The method of Claim 13, further comprising measuring the number of copies of a single-copy gene present in both cancerous and normal noncancerous cells and comparing the number of the single copy gene to the number of copies of a CAS gene, wherein, in cancerous cells, the number of copies of the CAS gene is higher than the number of copies of the single-copy gene.

16. The method of Claim 13, wherein the number of copies of a CAS gene is determined by a polymerase chain reaction that amplifies either a 5' portion of the CAS gene, a 3' portion of the CAS gene, or both 5' and 3' portions of the CAS gene.

17. The method of any one of Claims 13 to 16, wherein the cancer cells are from breast, colon, or lymph tissue.

18. A kit for utilizing the method of Claim 13, wherein said kit includes polynucleotides that serve as primers in a polymerase chain reaction for direct amplification of a nucleic acid coding for CAS protein and one or more reagents for performing a polymerase chain reaction or detecting a product of the polymerase chain reaction.

19. An isolated antibody that specifically recognizes CAS protein.

20. A kit for diagnosing cancer, said kit including an antibody according to Claim 19 and a label for indicating binding of said antibody to CAS protein.

21. The cDNA of any one of Claims 1-5, for use in preventing or reversing apoptosis in mammalian cells.

22. Use of the cDNA of any one of Claims 1-5 in the preparation of a medicament for preventing or reversing apoptosis in mammalian cells.

23. The antibody of Claim 19, for use in treating cancer in a mammal.

24. Use of the antibody of Claim 19 in the preparation of a medicament for treating cancer in a mammal.

25. A method for preventing or reversing apoptosis in a mammalian cell, comprising the steps of:
providing a cDNA according to any one of Claims 1-5 in a vector such that an antisense CAS transcript will be made in a cell under the regulation of DNA sequences contained within said vector; and
administering an effective amount of said vector to a mammalian cell thereby delivering said vector and CAS cDNA intracellularly.

26. A method of treating cancer in a mammal in need of treatment, comprising:

administering to the mammal an agent that decreases CAS protein activity in cells, wherein said agent is an antibody that specifically recognizes CAS protein or an antisense CAS gene sequence that inhibits CAS protein activity by decreasing expression of a CAS gene.

27. The method of Claim 26, wherein the agent is an antisense CAS gene sequence of the sequence of nucleotides 2100 to 2536 of SEQ ID NO:1 in a vector that includes genetic elements that regulate transcription of said antisense CAS gene sequence.

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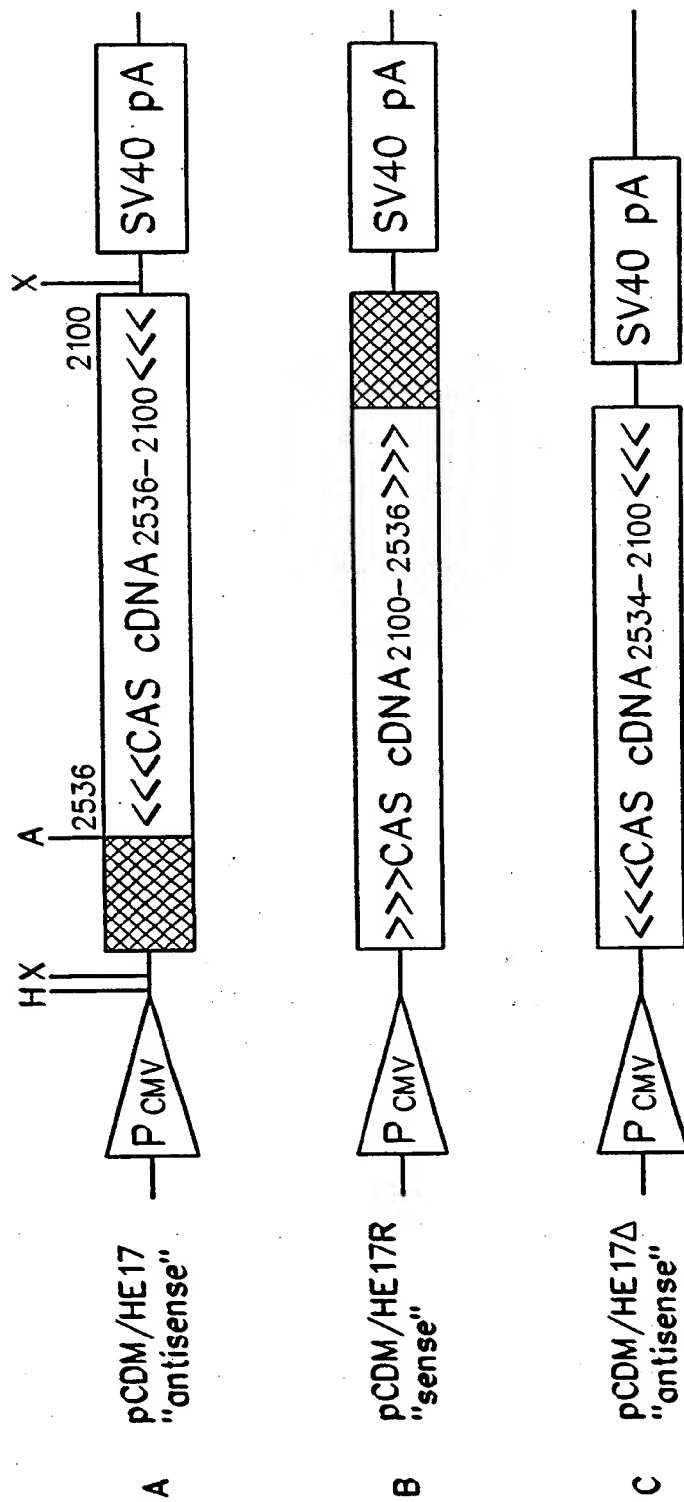


FIG. 1

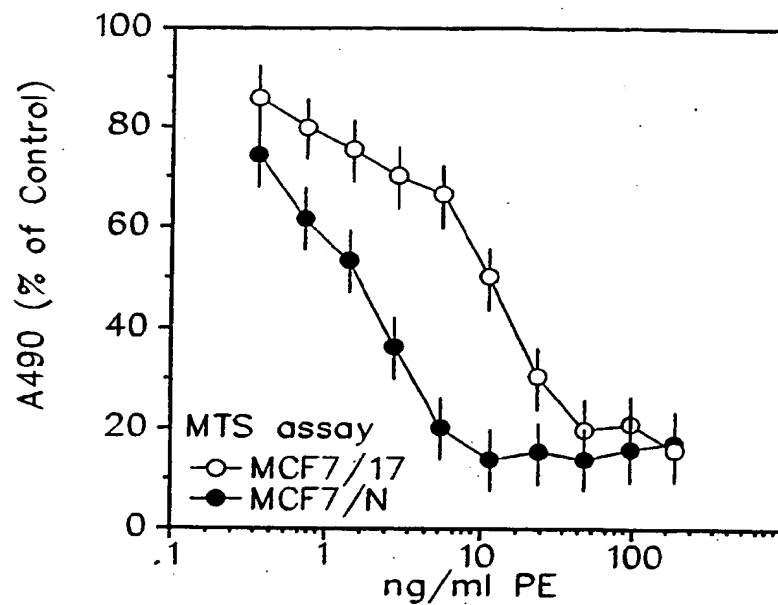


FIG. 2A

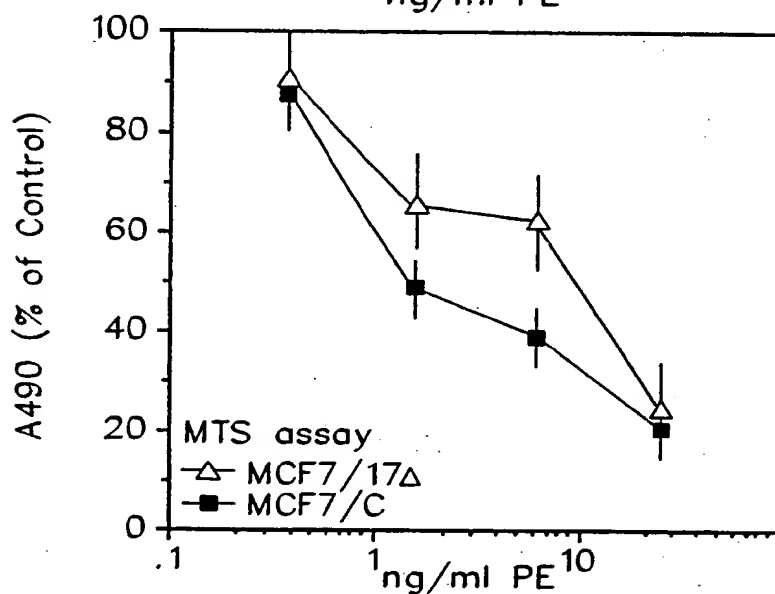


FIG. 2B

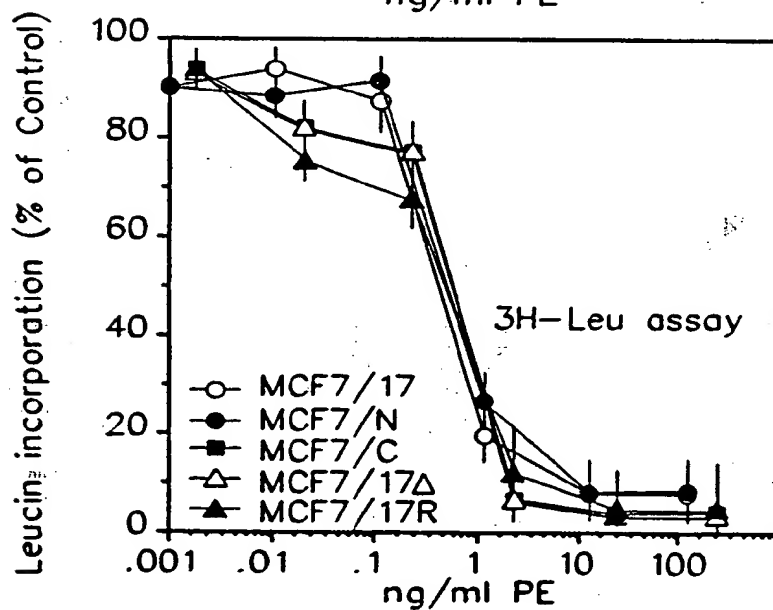
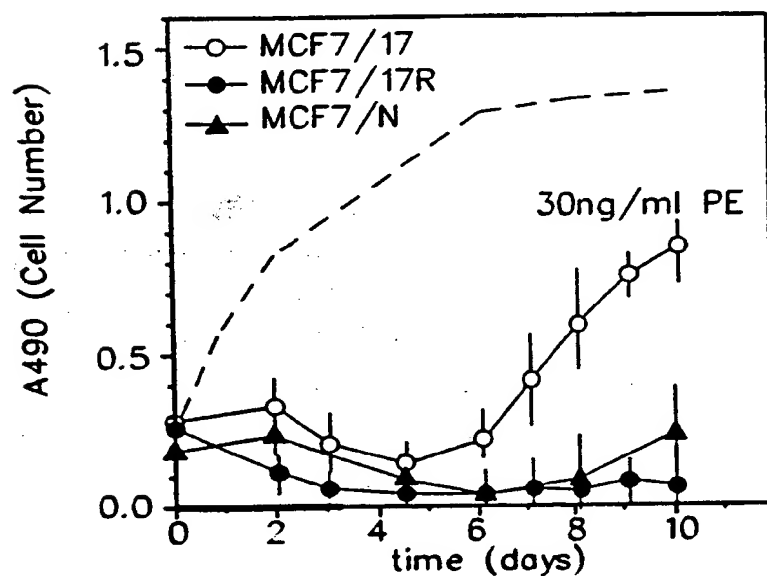
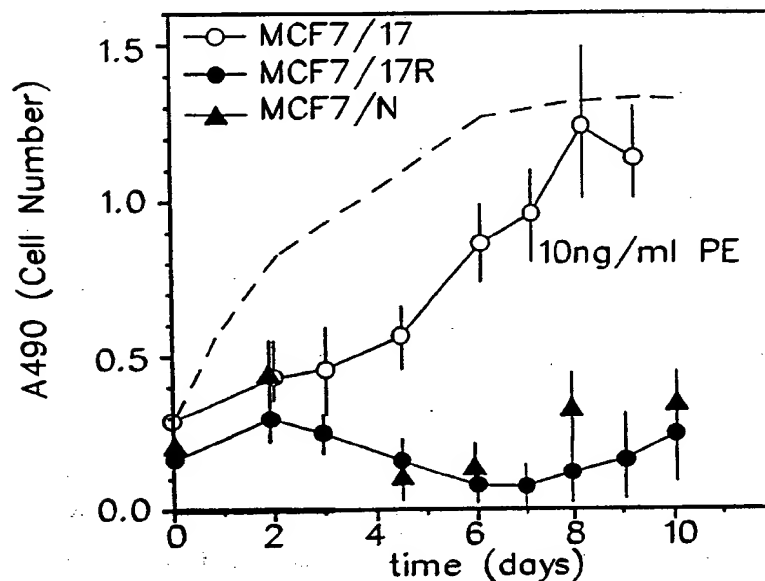
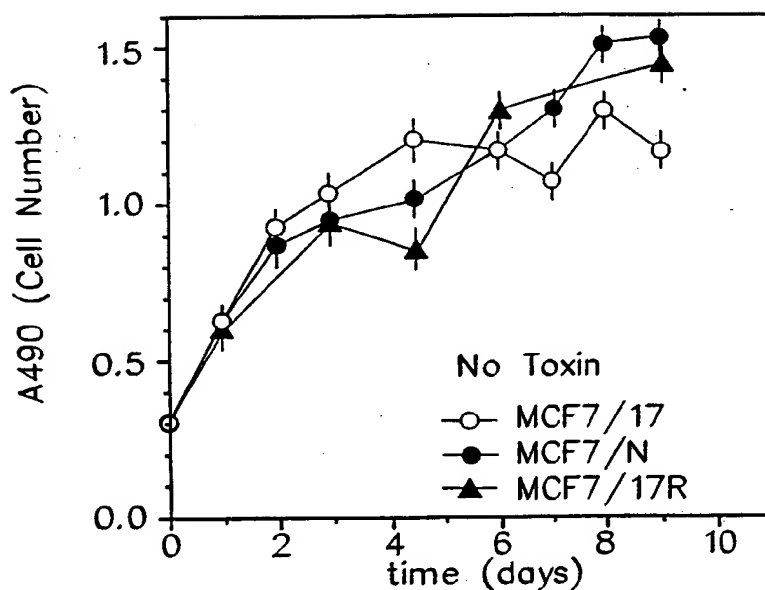


FIG. 2C



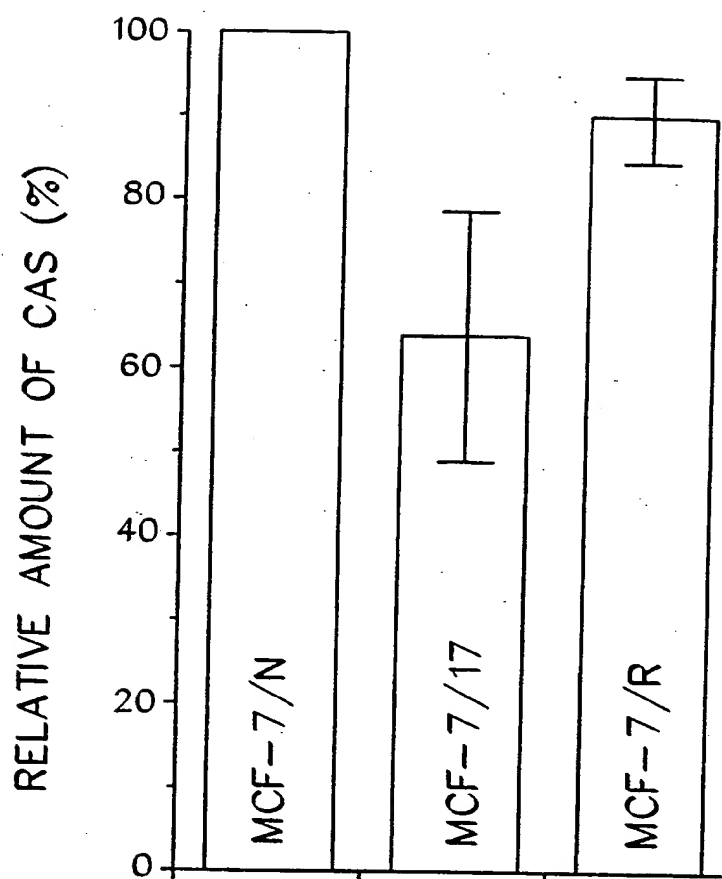


FIG. 3

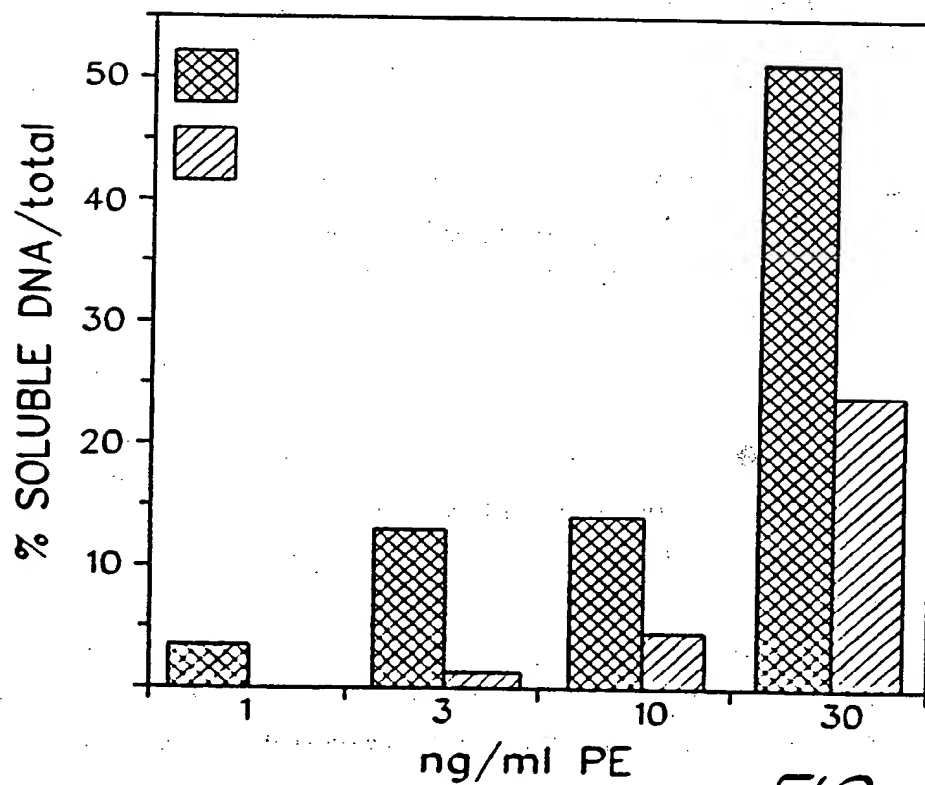


FIG. 4

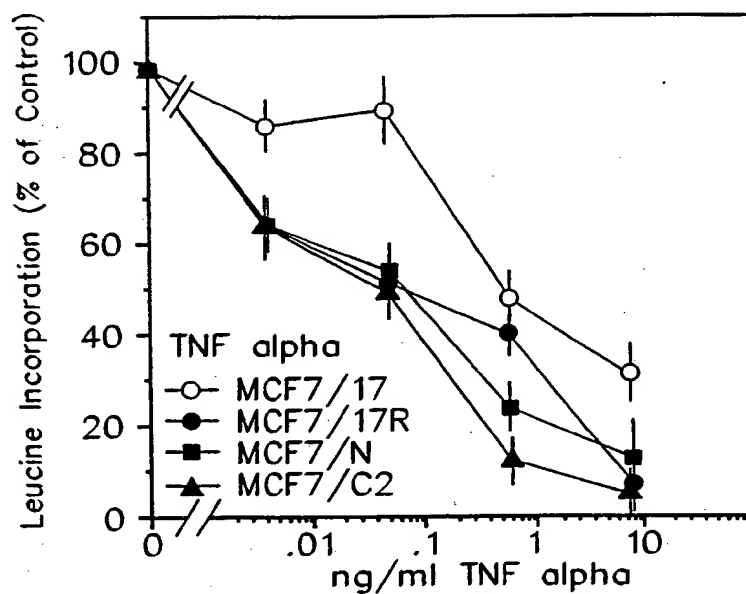


FIG. 5A

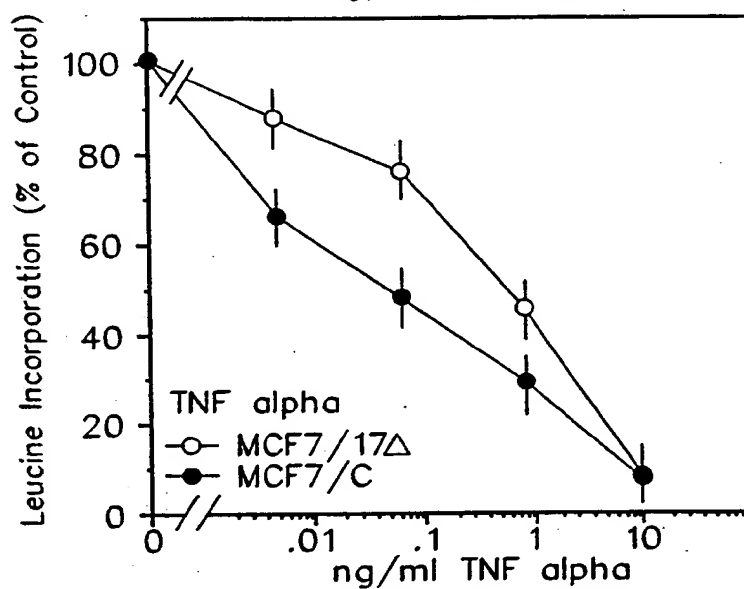


FIG. 5B

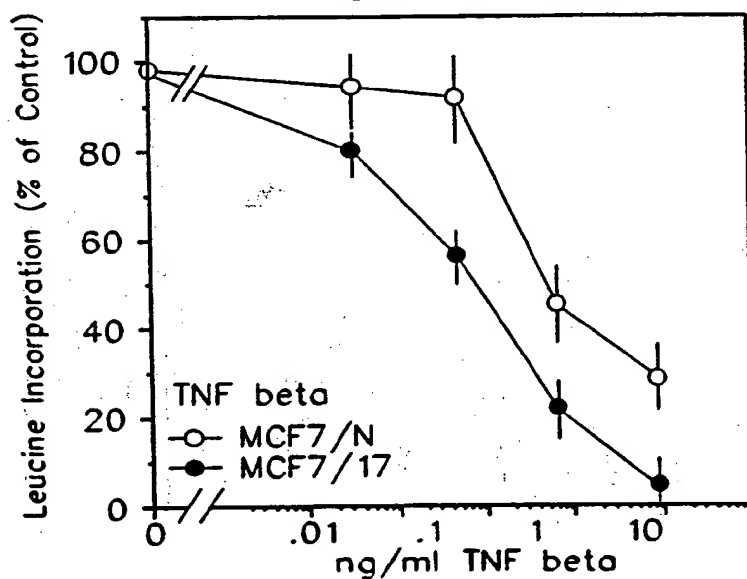


FIG. 5C

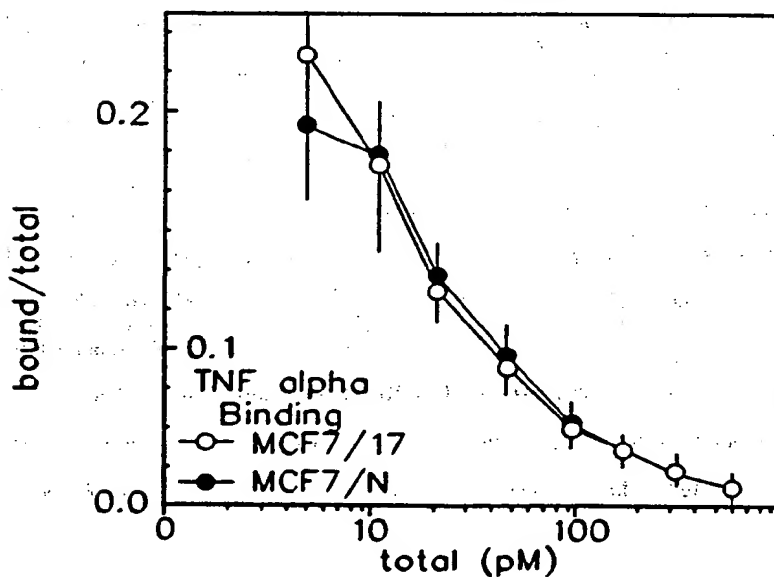
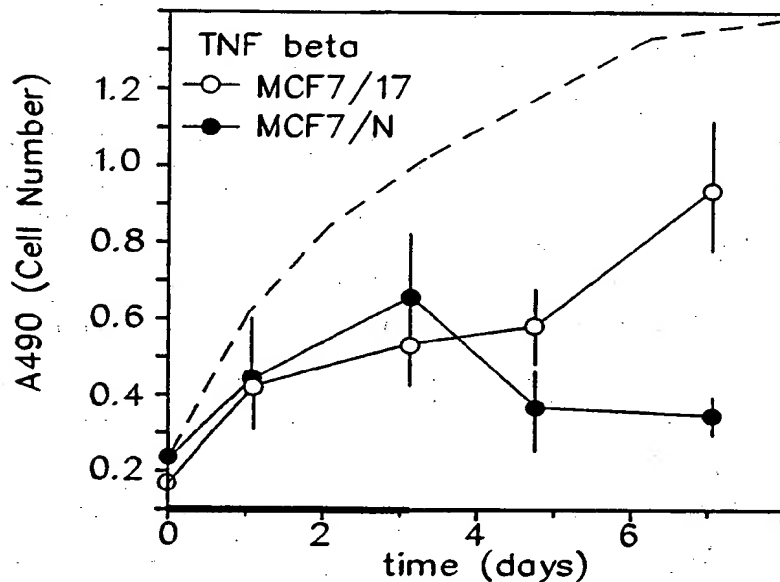
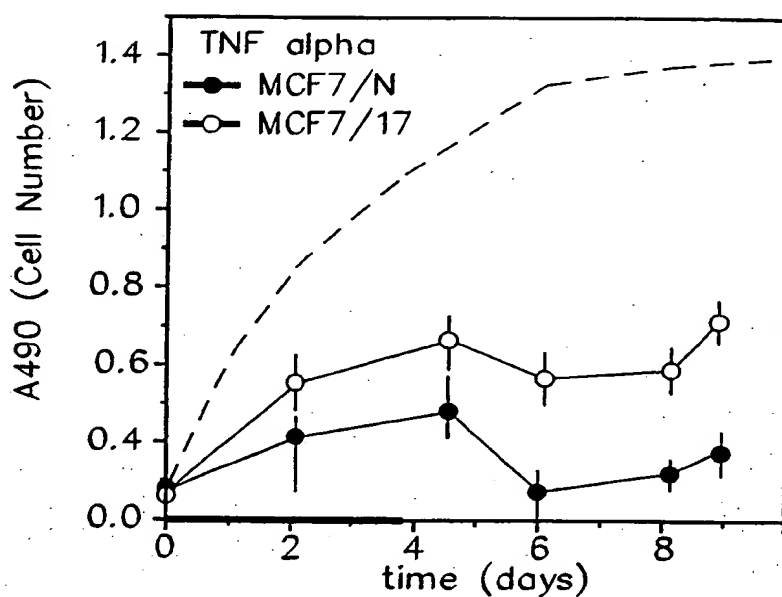


FIG. 6

FIG. 6A. CAS cDNA SEQUENCE

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ACTATATTTAAAGGAACTGGAGAATTGTTGAAGATGAACCAACAAAATTTGTGAAGCCGATCGA
GTGGCCATTAAAGCCAACATAGTGCACCTTGATGCTTAGCAGCCAGAGCAAATTCAGAAGCAGTT
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TCATTATTTAAAGATACCGTCATGAATTTAAGTCAAACGAGTTATGGACTGAAATTAAGCTTGT
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FIG. 6B. CAS PROTEIN SEQUENCE

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NFHTLLTLDN	KLLQTDDEEE	AGLLELLKSQ	ICDNAALYAO	KYDEEFQRYL	PRFVTAIWNL
LVTTGQEVKY	DLVSNAIQF	LASVCERPHY	KNLFEDQNTL	TSICEKVIVP	NMEFRAADEE
AFEDNSEEYI	RRDLEGSID	TRRRAACDLV	RGLCKFFEGP	VTGIFSGYVN	SMLQEYAKNP
SVNWKHKDAA	IYLVTSLSK	AQTQKHGITO	ANELVNLTEF	FVNHILPDLK	SANVNEFPVL
KADGIKYIMI	FRNQVPKEHL	LVSIPLLINH	LQAGSIVVHT	YAAHALERLF	TMRGPNNATL
FTAAEIAPFV	EILLTNLFKA	LTLPGSSENE	YIMKAIMRSF	SLLQEAIIPI	IPTLITQLTQ
KLLAVSKNPS	KPHFNHYMFE	AICLSIRITC	KANPAAVVNF	EEALFLVFTE	ILQNDVQEFI
PYVFQVMSLL	LETHKNDIPS	SYMALFPHLL	QPVWERTGN	IPALVRLQA	FLERGSNTIA
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SKTTKFIKSF	LVFINLYCIK	YGALALQEIF	DGIQPKMFGM	VLEKIIPEI	QKVSGNVEKK
ICAVGITNLL	TECPMMDTE	YTKLWTPLLQ	SLIGLFELPE	DDTIPDEEHF	IDIEDTPGYQ
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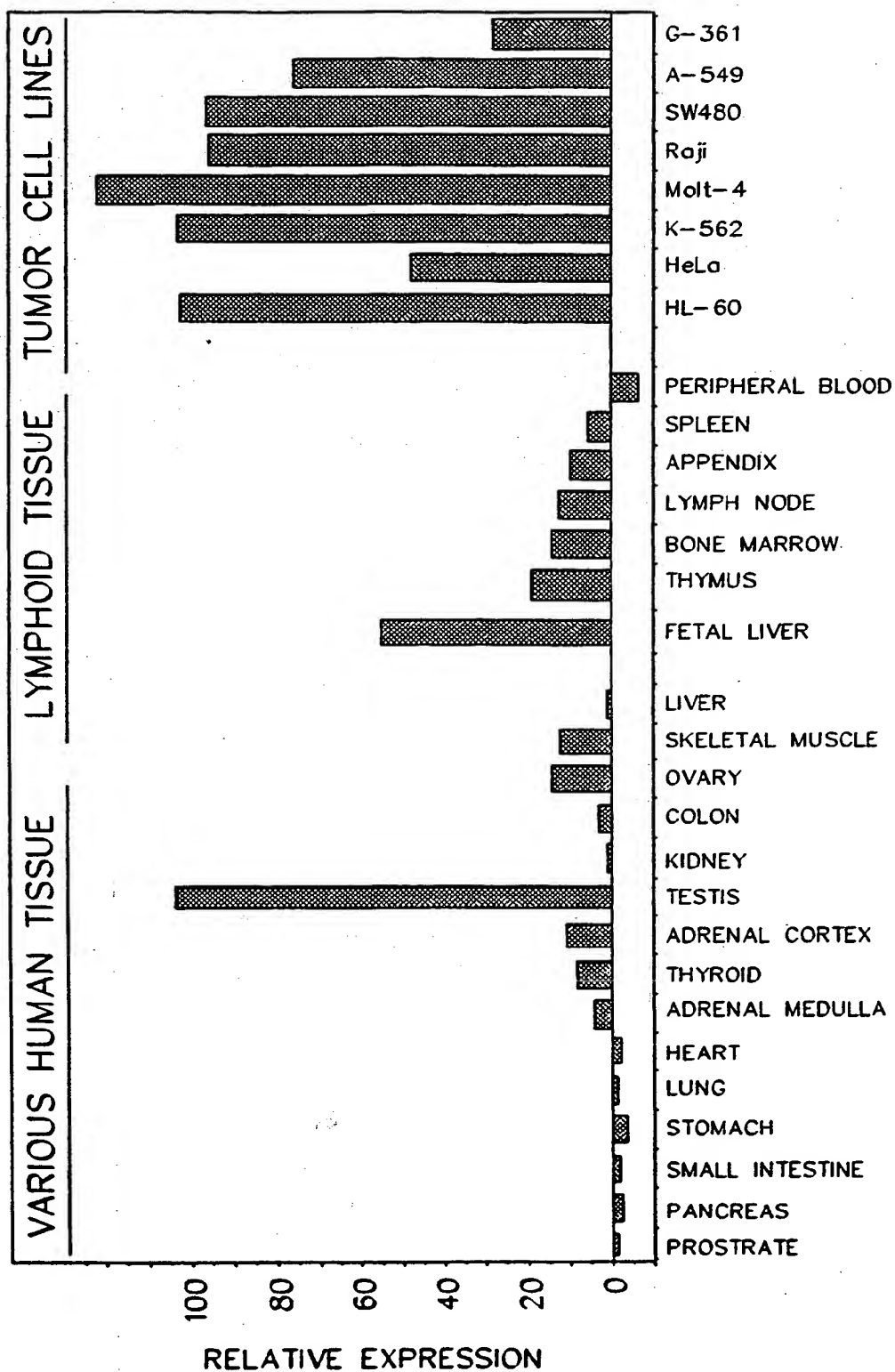


FIG. 7

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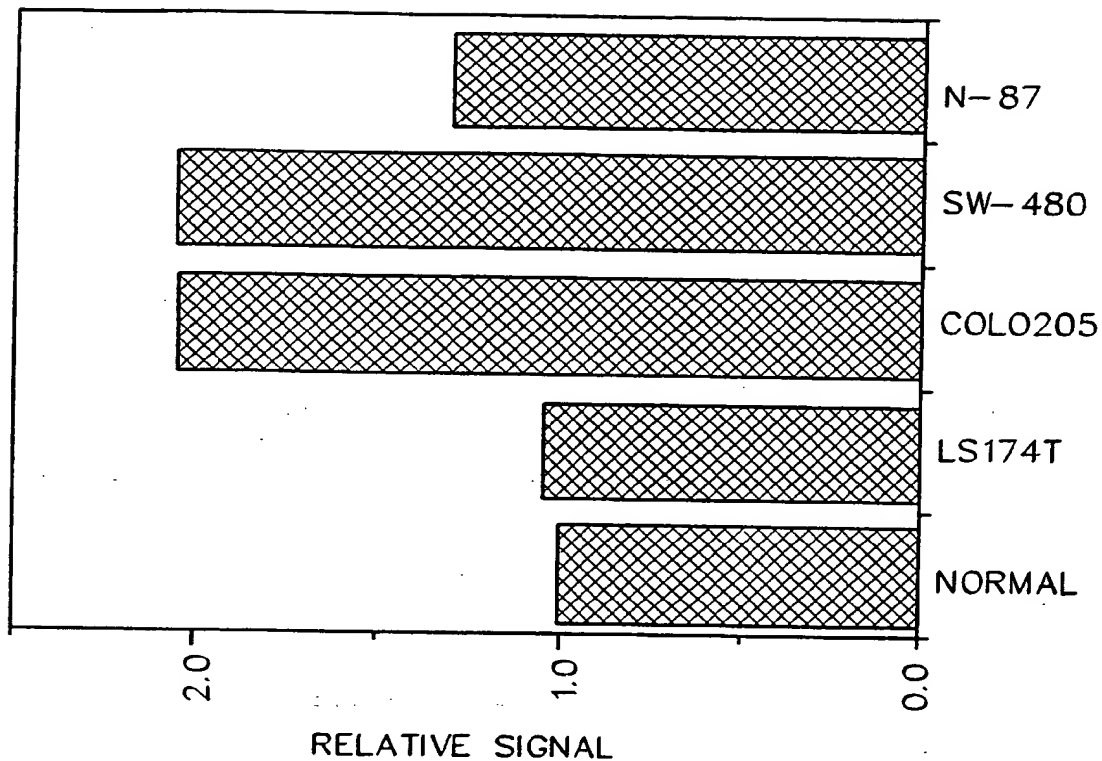


FIG. 8B

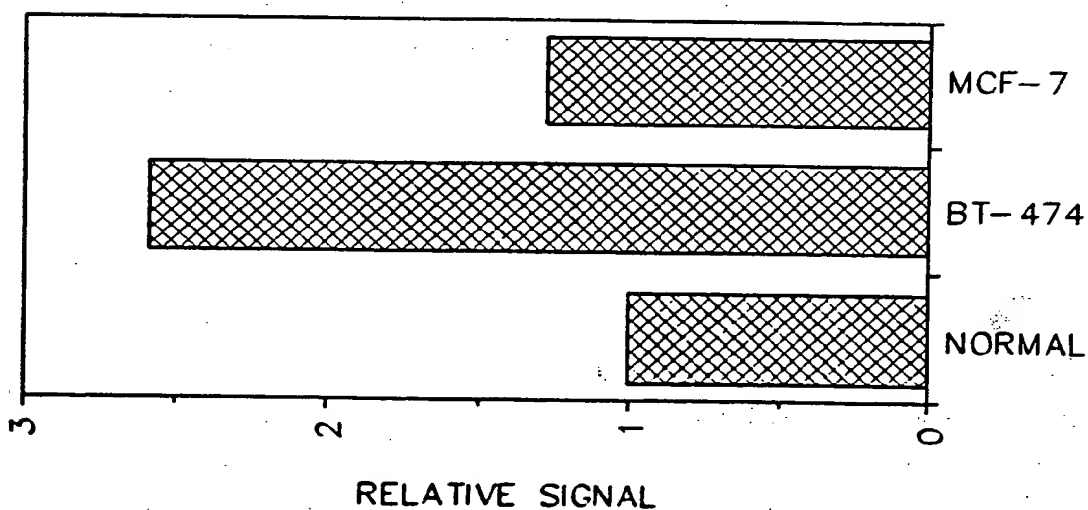


FIG. 8A

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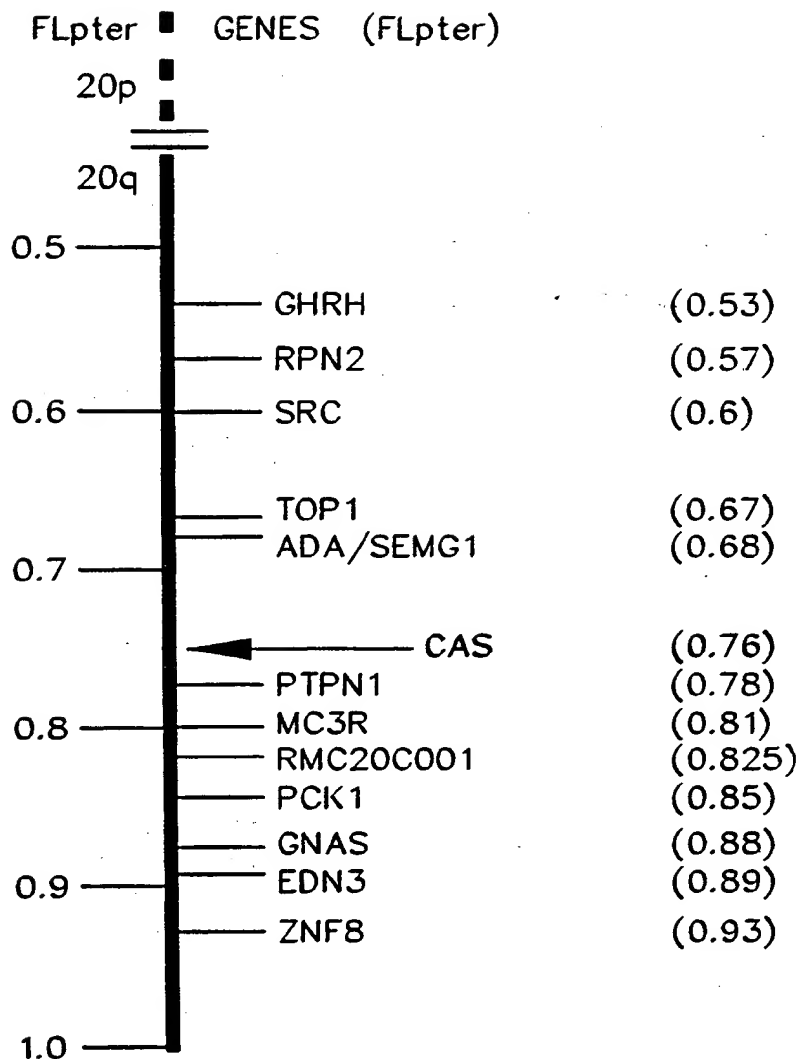


FIG. 9

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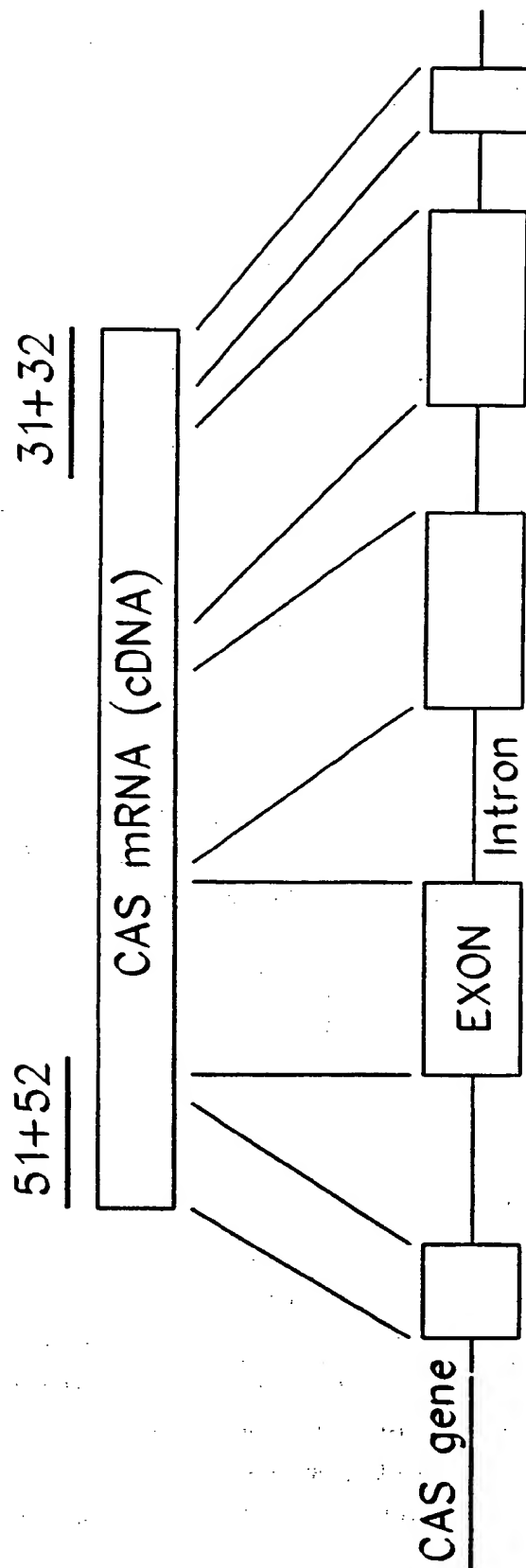


FIG. 10

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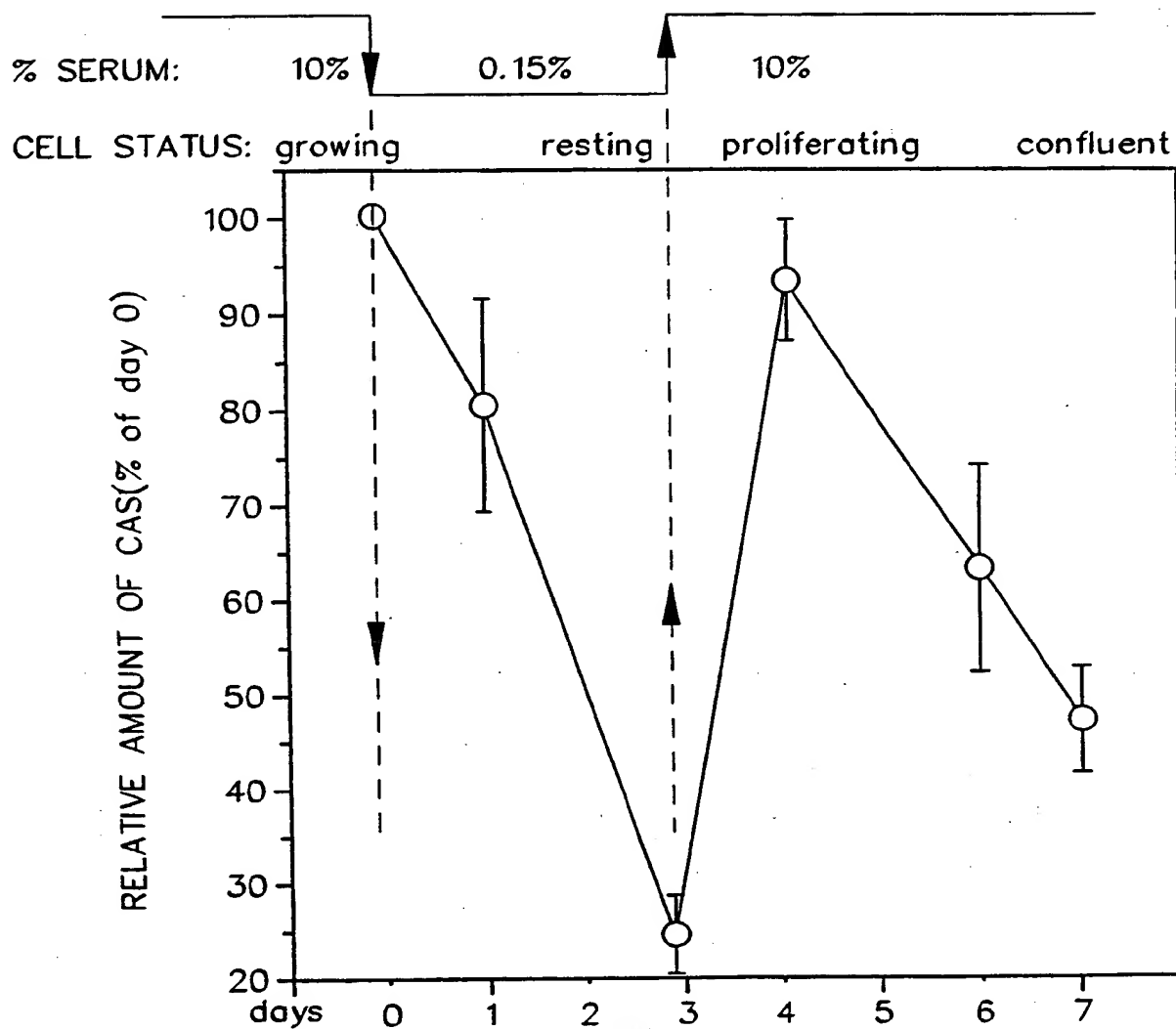


FIG. 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09927

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN, BIOSIS, MEDLINE, CANCERLIT, EMBASE, GENBANK, CAPLUS

search terms: apoptosis, programmed cell death, antisense, gene therapy, cancer cells

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P <u>Y,P</u>	BRINKMANN et al. Cloning and characterization of a cellular apoptosis susceptibility gene, the human homologue to the yeast chromosome segregation gene CSE1. Proc. Natl. Acad. Sci. USA. October 1995, Vol. 92, pages 10427-10431, see entire document.	1-7 <u>8-27</u>
A	BRINKMANN et al. Expression Cloning of cDNAs that Render Cancer cells resistant to Pseudomonas and Diphtheria Toxin and Immunotoxins. Mol. Med. January 1995, Vol. 1, No. 2, pages 206-216.	1-27
A	Cohen, J.J. Overview: Mechanisms of apoptosis. Immunology Today. 1993, Vol. 14, No. 3, pages 126-130.	1-27

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

06 SEPTEMBER 1996

Date of mailing of the international search report

02 OCT 1996

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KAREN HAUDA

Telephone No (703) 305-6608

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09927

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C07H 21/00; C07K 16/00; C12N 5/00, 15/63, 15/79, 15/00; A61K39/00, 38/00, 48/00

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/320.1, 240.2, 6, 7.1, 69.1, 172.3, 7.2, 7.9; 514/44, 2, 21; 935/62, 52, 55, 56, 57, 34, 70, 71, 34, 65; 536/23.1, 24.5; 424/93.1, 93.21

B. FIELDS SEARCHED

Minimum documentation searched

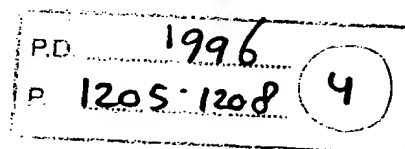
Classification System: U.S.

435/320.1, 240.2, 6, 7.1, 69.1, 172.3, 7.2, 7.9; 514/44, 2, 21; 935/62, 52, 55, 56, 57, 34, 70, 71, 34, 65; 536/23.1, 24.5; 424/93.1, 93.21



DB

PII: S0959-8049(96)00016-0



XP-002268751

Original Paper

DNA Amplification on Chromosome 3q26.1-q26.3 in Squamous Cell Carcinoma of the Lung Detected by Reverse Chromosome Painting

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Multiple genetic lesions have been reported in small cell lung carcinoma (SCLC), while considerably less information is available on squamous cell carcinoma (SCC). We used reverse chromosome painting to screen a total of nine SCCs for DNA amplifications. In three of the nine SCCs, hybridisation signals were found at chromosome region 3q26.1-q26.3, which does not contain any known oncogene. In one of the three SCCs, there were additional hybridisation signals at 1q, 5p and 6p21.1. The high frequency of a consistent amplification (3q26.1-q26.3) in SCC strongly indicates a novel gene at 3q26.1-q26.3 that is important in the pathology of SCC. Copyright © 1996 Elsevier Science Ltd

Key words: NSCLC, amplification, *in situ* hybridisation, chromosome 3
Eur J Cancer, Vol. 32A, No. 7, pp. 1205-1208, 1996

INTRODUCTION

DNA AMPLIFICATION has been reported for a wide spectrum of cancers and appears to contribute to the malignant phenotype [1, 2]. There is, however, no example of developmentally regulated gene amplification in mammals. In some instances, DNA amplification has been shown to be of prognostic significance, most notably amplification of the gene *NEU* in human breast cancer and the gene *NMYC* in neuroblastoma [3-6].

There is increasing evidence for gene amplification in non-small cell lung cancers (NSCLC) that account for 75-80% of all lung tumours [7, 8]. Double minutes (DMs) as cytogenetic manifestations of gene amplification were found in 77% of short-term cultures derived from NSCLC [7]. Southern blot analysis revealed amplification of several proto-oncogenes in NSCLC including *CMYC* and *KRAS2* [8-10]. There were also reports of amplifications of the topoisomerase II gene and the gene for parathyroid hormone-related protein (PTHrP) in NSCLC [8, 11]. Taken together, the emerging pattern of gene amplification in NSCLC appears to be rather complex, suggesting different amplified genes are involved in the pathogenesis of NSCLC. Cytogenetic analysis of NSCLC has consistently revealed an accumulation of multiple chromosome

alterations, indicating several independent genetic events as first shown for colon cancer [12-14].

Although the high incidence of DMs in NSCLC emphasises the crucial role of DNA amplifications in this tumour type, there is virtually no information on the chromosomal origin of these extrachromosomal amplification units. The genes known to be amplified in NSCLC are likely to account only for a small percentage of the total number of amplification events. To identify and localise novel amplification units in NSCLC, reverse chromosome painting was employed. Labelled genomic DNA from tumour cells and unlabelled sonicated DNA from normal cells were hybridised as a probe against a metaphase spread with normal male karyotype [15, 16]. Amplified DNA sequences in tumour DNA were indicated by intense fluorescence hybridisation signals.

Reverse chromosome painting has successfully been applied to identify amplified domains in human tumour cells [17, 18]. Most recently, 22 different amplified domains have been reported in small cell lung carcinomas (SCLCs) using comparative genomic hybridisation (CGH) [19]. The aim of this study was to identify amplified domains as yet unknown in squamous cell carcinoma (SCC) using reverse chromosome painting.

MATERIALS AND METHODS

Isolation and labelling of tumour DNA

Tissue samples of SCC of the lung were frozen in liquid nitrogen immediately after biopsy and stored at -70°C. Gen-

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omic DNA was isolated from the tumour samples by standard methods [20]. In brief, after proteinase K digestion at 55°C, proteins were extracted with chloroform and DNA was precipitated with isopropanol. Tumour DNA was labelled with biotin-16-dUTP by nick translation (Nick Translation System, Gibco BRL, Germany).

Isolation and preparation of DNA for competition

High molecular weight DNA was prepared from lymphocytes of normal karyotype (46XX) according to standard protocols [20]. DNA was sonicated and fragments of 200–500 bp were found by agarose gel electrophoresis.

Metaphase chromosome spreads

Metaphase chromosomes were prepared from peripheral blood lymphocytes (46XY) according to standard methods [21] and suspensions were stored in methanol:acetic acid (3:1) at –20°C.

In situ hybridisation

Biotin-labelled tumour DNA (150–200 ng) was mixed with sonicated normal DNA at a ratio of 1:1 and combined with 40 µg COT-1 DNA in a total hybridisation volume of 2.5 µl. Alternatively, labelled tumour DNA was hybridised in the presence of a 100-fold excess of COT-1 DNA without competition by sonicated normal DNA. The probes were denatured at 75°C for 5 min, reannealed at 37°C for 10–20 min and hybridised to normal male metaphase chromosomes at 37°C overnight. Prior to hybridisation, slides were treated with RNase A for 1 h and pepsin for 10 min. After dehydration in ethanol, chromosomes were denatured in a 70% formamide solution at 80°C for 2 min and again dehydrated by three cold ethanol washes (70%, 80%, 96%).

Detection

Following washes in a 50% formamide solution at 45°C and in 0.1 × SSC at 60°C, biotinylated probes were detected using avidin conjugated to fluorescein isothiocyanate. One round of amplification was performed by using goat anti-avidin antibodies. Slides were counterstained with 4,6-diamino-2-phenyl-indol (DAPI) and mounted in fluorescence-antifading buffer. Fluorescence signals were visualised in a Zeiss microscope, captured by image integration with a Photometrics camera (CE 200A Camera), analysed and documented with the program ISIS3, version 1.53 of MetaSystems. To be counted as positive, hybridisation signals were required in at least 45 of the 50 metaphases analysed.

RESULTS

To detect DNA amplifications, a total of nine SCCs were analysed by reverse chromosome painting. Three specimens were histopathologically characterised as WHO (World Health Organisation) grade I and five specimens as WHO grade III tumours. Tumour DNA was labelled by biotin-16-dUTP mixed with COT-1 DNA and unlabelled sonicated blood DNA, and hybridised against metaphase spreads with normal male karyotype. Defined fluorescence hybridisation signals were found in three of the nine cases, indicating a high frequency of gene amplification in SCCs. There was no difference between hybridisations performed with and without unlabelled genomic DNA. In all three cases, an amplification event was localised within region 3q26.1–q26.3. In one of the three SCCs, there were additional hybridisation signals at 1q,

5p and 6p(21.1). Our results are strongly indicative of an amplified gene at 3q26.1–q26.3 which is likely to be of importance in the pathology of SCC. Figure 1 shows a representative reverse chromosome painting experiment demonstrating the hybridisation signal at 3q26.1–q26.3. To our knowledge, there are no oncogenes within chromosome region 3q26.1–q26.3, indicating a novel amplified gene residing within this chromosome region. The results of the reverse chromosome painting experiments are summarised in Table 1, including pathological staging of the tumours.

DISCUSSION

Our data demonstrate a specific involvement of a region on chromosome 3q in SCC. In previous studies, many genetic alterations have been associated with lung cancer, the majority with SCLC [22, 23]. In both SCLC and NSCLC, the most frequent chromosomal alterations are deviations of chromosome 3, with loss of the short arm as the most consistent finding [22, 24–26]. Previously, Ried and colleagues [19] reported a simultaneous over-representation of the long arm of chromosome 3 in primary SCLC. The loss of the short arm of chromosome 3, together with the over-representation of the

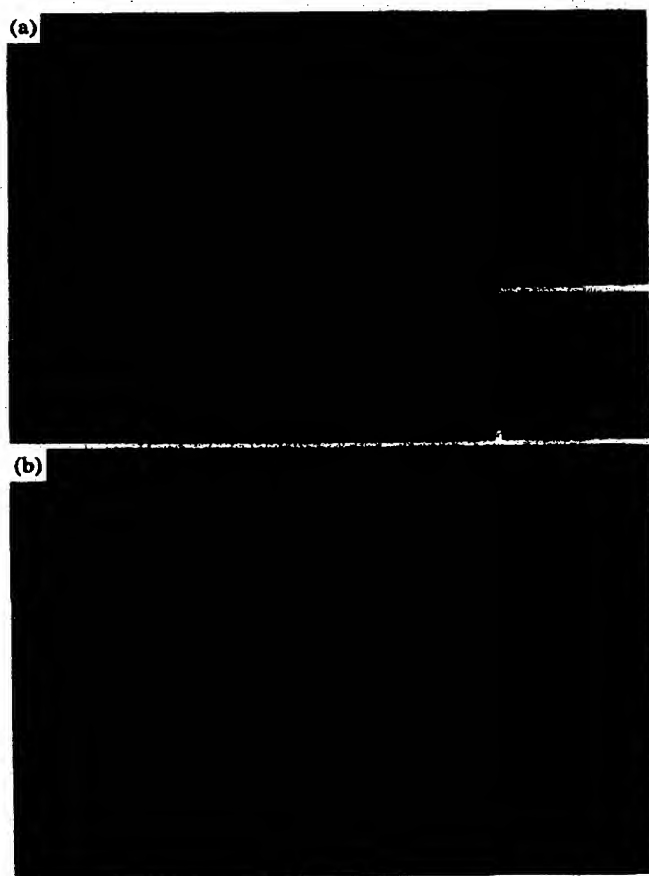


Figure 1. (a) Reverse chromosome painting using fluorescence-labelled DNA from tumour L26. Hybridisation signals were identified at 3q26.1–q26.3 (arrows). (b) Metaphase spread shown in (a), counterstained with 4,6-diamino-2-phenyl-indol. The inset to (a) depicts a second case of reverse chromosome painting analysis. Hybridisation signals at 3q26.1–q26.3 were identified using DNA from tumour L1.

Table 1. Clinicopathological features of nine patients with lung cancer and chromosomal location of amplified DNA sequences

Case	Age	Sex	Pathological stage				Amplification
			T	N	M	Stage	
L1	67	M	4	2	0	IIIb	3q26.1-q26.3
L6	69	M	2	0	0	I	-
L10	65	M	2	0	0	I	1q,3q26.1-q26.3,5p,6p21.1
L12	68	M	3-4	x	0	IIIb	-
L13	65	M	2	0	0	I	-
L22	75	M	4	2	0	IIIb	-
L26	65	M	4	2	0	IIIb	3q26.1-q26.3
L27	54	M	3-4	2	0	IIIb	-
L38	73	M	3-4	x	x	nd	-

T, tumour; M, metastasis; N, node.

long arm, is consistent with the idea of isochromosome 3q in a subset of SCLC.

Although alterations of chromosome 3 appear to be a hallmark of lung tumours, the underlying molecular mechanisms are likely to differ in SCLC and NSCLC. In contrast to SCLC, allelic loss is frequently found not only for the short arm of chromosome 3, but also for the long arm [27]. In addition, alleotyping indicates differences in the tumorigenic mechanisms of SCC and adenocarcinoma as the major histological subtypes of NSCLC [27, 28]. Furthermore, our study failed to reveal an over-representation of the entire long arm in any of the analysed SCCs, but indicated a specific amplification site at 3q26.1-q26.3. In SCLC, there is only one case reported to carry a specific amplification on 3q [19]. However, the regional assignment of this amplified domain that falls within 3q27-28 differs from the localisation of the amplification identified in SCC (3q26.1-q26.3). Taken together, these data indicate that a specific amplified gene within 3q26 is likely to play a causative role in the pathology of SCC, but not necessarily in adenocarcinoma and SCLC.

The high incidence of DMs in 77% of NSCLC provides further evidence for DNA amplification as an important mechanism in tumour development [7]. As amplifications of single genes are not necessarily demonstrated by reverse chromosome painting, the percentage of NSCLC carrying an amplified gene at 3q26.1-q26.3 might be higher than indicated in our study (3/9 cases). As shown by Joos and colleagues [17], the detection depends not only on the amplification level, but also on the extension of the amplified domain.

Importantly, the amplification at 3q26.1-q26.3 was found in all SCCs that were identified as carrying an amplified domain by reverse chromosome painting. This finding strongly indicates a key role for this chromosomal region in the development of SCC.

Although there are few reports on specific amplified domains in NSCLC, e.g. *PTHRT* and *KRAS*, none of these genes reside within region 3q26.1-q26.3. To our knowledge, no oncogenes have been mapped on this region. Further studies are warranted to delineate which specific genes are amplified in SCC at 3q26.1-q26.3.

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